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13. ABSTRACT (Maximum 200 Words)

Proliferation and differentiation of normal breast epithelial cells are regulated by activation of the cellular tyrosine kinase machinery upon coordinated cellular stimulation through growth factor receptor tyrosine kinases and extra-cellular matrix receptor-induced activation of focal adhesion kinase FAK. This proposal is designed to investigate a novel hypothesis that Cbl provides, which has become established as a negative regulator of growth factor receptors, attenuates FAK-dependent growth signals in mammary epithelial cells. Given the recent findings that Cbl functions as ubiquitin ligase towards tyrosine kinases, we have been examining the possibility that Cbl regulates FAK signaling by targeting it for degradation directly and/or by targeting Src-family kinases that function as upstream activators of FAK. The work reported here describes FAK mutants that appear to be unable to interact with Cbl tyrosine kinase-binding domain and their ability to alter actin cytoskeleton. Together with the demonstration of Src-family degradation and negative regulation by Cbl, the present studies establish FAK as a target of Cbl. Further studies based on these novel findings are likely to provide important insights into the regulation of proliferation signals in breast cancer cells.

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Regulation of FAK Signaling in Mammary Epithelial Cells by Cbl Proto-Oncogene Product

Introduction:

The experiments proposed in this application were designed to test a unique hypothesis that the proto-oncogene product Cbl down-regulates proliferation signals in mammary epithelial cells by concurrently targeting the focal adhesion kinase, FAK, in addition to its better defined ability to target growth factor receptors of the ErbB family. Genetic studies, initially in C. elegans and Drosophila systems and recently using mouse knock-outs, as well as extensive biochemical studies have established Cbl as a negative regulator of tyrosine kinases. At the time of this application, the tyrosine kinase-binding (TKB) domain of Cbl had been shown to be crucial for Cbl function, and initial pull-down experiments indicated that the TKB domain of Cbl interacts with FAK. These findings provided a rationale for the proposed analyses to identify the TKB domain binding sites on FAK and to introduce mutations in FAK to abrogate its ability to interact with the Cbl TKB domain in order to investigate the potential regulatory role of Cbl for FAK. Further studies in our laboratory demonstrated that Cbl interacts with and regulates the function of Src-family kinases, which in turn are known to be essential for the activation of FAK. Thus, it became clear that Cbl might regulate FAK both directly as well as indirectly via its negative regulatory effects on Src-family kinases. Transfection approaches and analysis of cell lines from Cbl+/+ wildtype and Cbl-/- knockout mice further supported the original hypothesis that Cbl controls FAK signaling in mammary epithelial cells. Studies carried out during this training award have provided defined the Cbl interaction sites on FAK, and have provided initial evidence that interactions mediated via these sites control FAK activity. These studies also highlighted technical hurdles that need to be overcome to fully investigate the role of the novel Cbl-dependent FAK regulatory pathway in the control of mammary cell proliferation. Understanding the mechanisms of this novel biochemical pathway to control mammary epithelial cell proliferation and migration is likely to provide crucial insights into breast cancer pathogenesis and may help in the design of newer forms of treatment.

Body:

Studies carried out in the course of this trainee award helped provide advanced training for the original awardee (Dr. Patrice Douillard) and his subsequent replacements (Dr. Stephen Donoghue and Dr. Amiya Ghosh) after he moved to a Pharmaceutical position in his parent country, France. Our studies have delineated a potentially significant role of the putative Cbl-binding site on FAK in controlling its function. Furthermore, Cbl became established as a negative regulator of Src-family kinases that form essential upstream activators of FAK. As reported previously, some of the approaches that we attempted met with technical difficulties resulting in slower progress. However, the important new insights gained through our studies have provided a novel mechanism for the control of FAK-mediated signaling, and provided a basis for further studies to unravel the role of this pathway in mammary epithelial cell proliferation and breast cancer.

Analyses of Cbl-FAK interaction: As reported previously, our studies using GST fusion protein pull-down method as well as coimmunoprecipitation approaches demonstrated

that Cbl and FAK interact with each other. One prominent mechanism of this interaction emerged to be the direct binding of the Cbl TKB domain to FAK. Analysis of a with a TKB domain mutant of Cbl strengthened this observation but also indicated that additional mechanisms for Cbl-FAK association were likely, as the mutant retained its ability to associate with FAK. As the Cbl TKB domain recognizes phospho-tyrosine-containing motifs on target proteins, we investigated the possible motifs on FAK that could mediate such an interaction. These studies showed that two tyrosine phosphorylation sites on FAK, pY397 and pY861, were the likely candidates for Cbl TKB domain binding. Previous studies have indicated that FAK Y397 is an auto-phosphorylation site and mediates the recruitment of Src-family kinases such as Src, Fyn and Yes, by providing a docking site for their SH2 domains (Sieg DJ et al. Nat. Cell Biol. 2:249-256, 2000; Nakamura K et al. Oncogene 20:2626-2635, 2001; Abu-Ghazaleh R et al. Biochem. J. 360:255-264, 2001). Once Src-family kinases are recruited, they enhance the phosphorylation of FAK on other sites: Y861 is a prominent in vitro Src-induced phosphorylation site as well as a major in vivo phosphorylation site on FAK (Abu-Ghazaleh R et al. Biochem. J. 360:255-264, 2001). Furthermore, phosphorylated Y861 functions to help in further recruitment of the Src-family kinases, although the mechanisms of this interaction are less clear (Abu-Ghazaleh R et al. Biochem. J. 360:255-264, 2001). Thus, these findings raised a strong possibility that the Cbl TKB domain binding to FAK may be SFK-dependent. Using co-transfection analyses with FAK or its mutants and Src-family kinase Fyn, we showed that this was the case.

Using a transient transfection system in Hela cells, we demonstrated that mutations of Cbl TKB domain-binding sites on FAK markedly alter its ability to change actin cytoskeleton of a cell. The altered morphology of cells expressing the FAK-Y861F mutant (rounded, start-shaped or smaller cells with short processes, a high concentration of actin near the cell boundary and loss of stress fibers) was reminiscent of that observed in FAK-/- cells (Sieg D. et al. J. Cell Sci. 112:2677-2691, 1999) (Fig. 9D and E. We are now following up these observations in FAK-deficient cell and human mammary epithelial cells. These analyses are being pursued in the context of EGFR stimulation. As reported earlier, we have identified and characterized the appropriate human mammary epithelial cell lines for these studies and have established conditions of their stimulation, biochemical analysis and cell migration.

Cbl regulation of Src-family kinases, upstream controllers of FAK: Given the essential role of Src-family kinases in FAK-dependent cell migration (Klinghoffer RA, et al. EMBO J. 18:2459-2471, 1999), and the ability of Cbl to downregulate Src-family kinases (Andoniou et al. Molecular and Cellular Biology, 20:851-867, 2000), we have hypothesized that Cbl might influence FAK signaling indirectly, via ubiquitin-dependent degradation of Src-family kinases. Therefore we initiated studies to investigate how Cbl regulates Src-family kinases. This work has revealed that Cbl induces the degradation of Src-family kinases Fyn and Lck by their ubiquitination-dependent targeting to the proteasome (see attached publications, Rao et al. and Rao et al). Further analyses have revealed that Cbl TKB domain, the proline-rich region as well as its phosphorylation contribute to Cbl-Fyn association which in turn determines Fyn degradation (see attached publication, Ghosh et al). The Fyn and Cbl mutants generated in these studies, together with FAK mutants isolated above, will provide crucial reagents to further investigate the novel regulatory pathway to control signaling through FAK.

Control of EGFR function by Cbl-mediated ubiquitinylation: In collaborative studies with other members of the laboratory, we have also examined the role of Cbl in the downregulation of EGFR, a prominent receptor tyrosine kinase linked to FAK activation and control of cell migration in breast cancer. We demonstrated that endogenous Cbl was essential for ligand-induced ubiquitinylation and efficient degradation of EGFR. Further analyses using CHO cells with a temperature-sensitive defect in ubiquitinylation confirmed a crucial role of the ubiquitin machinery in Cbl-mediated EGFR degradation. However, internalization into early endosomes did not require Cbl function or an intact ubiquitin pathway. Confocal immunolocalization studies indicated that Cbl-dependent ubiquitinylation plays a critical role at the early endosome to late endosome/lysosome sorting step of EGFR downregulation. These findings establish Cbl as the major endogenous ubiquitin ligase that is responsible for EGFR degradation, and show that the critical role of Cbl-mediated ubiquitinylation is at the level of endosomal sorting, rather than at the level of internalization. These findings provide critical new insights into our understanding of ErbB receptor downregulation (see attached publication, Duan et al).

Overall, the recent studies provide strong support for the role of Cbl in controlling FAK function. Given the important role of Src-family kinases and FAK in cell migration and proliferation, these studies are likely to provide important new insights into pathogenesis of breast cancer.

Key Research Accomplishments:

- Established the in vivo association between Cbl and FAK and identified a TKB-dependent and a TKB-independent mechanism for such association.
- Obtained FAK mutants with potential defects in Cbl binding and partially characterized these.
- Established a modified cell migration assay to assess the role of Cbl in FAK-dependent cell migration.
- Demonstrated a defect in PDGF, LPA and serum-induced cell migration in Cbl-/- cells as compared to Cbl+/+ cells.
- Demonstrated altered focal adhesions and actin stress fibers in Cbl-/- cells as compared to Cbl+/+ cells.
- Demonstrated the role of Cbl ubiquitin ligase activity in the regulation of Src-family kinase Fyn, which is upstream of FAK in integrin-dependent focal adhesion signaling.
- Established the mammary epithelial cell stimulation conditions to induce FAK phosphorylation on the Cbl TKB domain-binding site pY861.
- Established retroviral FAK expression construct.

- Demonstrated a defect in actin cytoskeleton upon transfection of Cbl non-binding FAK mutants.

- Established that Cbl regulation of Src-family kinases is mediated by ubiquitin-dependent proteasome targeting, and characterized the nature of molecular interactions between Cbl and Src-family kinases.

Reportable Outcomes:

Publications:

- Rao N, Ghosh AK, Zhou P, Ota S, Andoniou CE, Douillard P, Band H. An essential role of ubiquitination in Cbl-mediated negative regulation of the Src-family kinase Fyn. **Signal Transduction** 2002; 1-3:1-11.

- Rao N, Miyake S, Douillard P, Dodge I, Fernandes N, Druker B, Band H. Negative regulation of Lck tyrosine kinase by Cbl ubiquitin ligase. **Proc. Natl. Acad. Sci. USA.** 2002; 99:3794-3799.

- Ghosh AK, Rao NL, Lakku Reddi A, Duan L, Band V, Band H. Biochemical basis for the requirement of kinase activity in Cbl-dependent ubiquitinylation and degradation of a tyrosine kinase. In Preparation for Submission.

- Duan L, Miura Y, Dimri M, Majumder B, Dodge IL, Lakku Reddi A, Ghosh AK, Fernandes N, Zhou P, Mullane-Robinson K, Rao N, Donoghue S, Rogers RA, Bowtell D, Naramura M, Gu H, Band V, Band H. Cbl-mediated ubiquitinylation is required for lysosomal sorting of EGF receptor but is dispensable for endocytosis. **J. Biol. Chem.** 2003 May 18 [Epub ahead of print].

- Duan L, Miura Y, Dimri M, Majumder B, Dodge I, Lakkureddi A, Ghosh A, Fernandes N, Zhou P, Mullane-Robinson K, Rao N, Donoghue S, Bowtell D, Gu H, Naramura M, Band V, Band H. Cbl-mediated ubiquitination is required for lysosomal sorting of EGF receptor but is dispensable for endocytosis. Poster presentation at the 2002 Research fellows' Poster Session, Brigham and Women's Hospital, Boston.

- Donoghue S, Douillard P, Xiong W-C, Parsons JT, Band H. Regulation of FAK Signaling by Cbl. Poster presentation at the Era of Hope Meeting, Department of Defense Breast Cancer Research Program. Orange County Convention Center, Orlando, Florida. September 25-28, 2002 (Abstract P3-10).

Reagents:

- FAK expression construct with defects in Cbl TKB domain binding

- Mutant Fyn and Lck constructs with defined domain mutations.

- Cbl-/- cells with defective cell migration response

Funding applied for based on this work:

The work carried out under this award was part of the background and preliminary studies for an NIH RO1 application (Molecular Control of EGF Receptor Down-Regulation) submitted by Dr. Band to the NIH in February 2002; this application has been funded now. The studies carried out here also are part of preliminary data for new NIH RO1 grant submitted by Dr. Band (Endosomal ErbB receptor and Src signaling in Cancer) that is currently under review.

Manuscripts included:

- Rao N, Ghosh AK, Zhou P, Ota S, Andoniou CE, Douillard P, Band H. An essential role of ubiquitination in Cbl-mediated negative regulation of the Src-family kinase Fyn. **Signal Transduction** 2002; 1-3:1-11.

- Rao N, Miyake S, Douillard P, Dodge I, Fernandes N, Druker B, Band H. Negative regulation of Lck tyrosine kinase by Cbl ubiquitin ligase. **Proc. Natl. Acad. Sci. USA.** 2002; 99:3794-3799.

- Ghosh AK, Rao NL, Lakku Reddi A, Duan L, Band V, Band H. Biochemical basis for the requirement of kinase activity in Cbl-dependent ubiquitinylation and degradation of a tyrosine kinase. In Preparation for Submission.

- Duan L, Miura Y, Dimri M, Majumder B, Dodge IL, Lakku Reddi A, Ghosh AK, Fernandes N, Zhou P, Mullane-Robinson K, Rao N, Donoghue S, Rogers RA, Bowtell D, Naramura M, Gu H, Band V, Band H. Cbl-mediated ubiquitinylation is required for lysosomal sorting of EGF receptor but is dispensable for endocytosis. **J. Biol. Chem.** 2003 May 18 [Epub ahead of print].

Conclusions:

In conclusion, studies carried out under this trainee award have helped establish a novel interaction between Cbl ubiquitin ligase and focal adhesion kinase, a critical component of signaling initiated by combined activation of receptor tyrosine kinases and integrins with direct relevance to breast cancer. Our studies have established that direct binding of Cbl TKB domain to tyrosine phosphorylation sites in FAK provides on mechanism of Cbl-FAK interaction. A second mode of their interaction appears to be indirectly mediated by Src-family kinases. Src-family kinases themselves serve as targets of negative regulation by Cbl. Overall, our studies have helped identify a new mode of regulating FAK, a critical component of cell proliferation and migration in normal and cancerous cells.

Signal Transduction 2002, *1-3*, 1–11

An essential role of ubiquitination in Cbl-mediated negative regulation of the Src-family kinase Fyn

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The Cbl family of ubiquitin ligases function as negative regulators of activated receptor tyrosine kinases by facilitating their ubiquitination and subsequent lysosomal targeting. Here, we have investigated the role of Cbl ubiquitin ligase activity in the negative regulation of a non-receptor tyrosine kinase, the Src-family kinase Fyn. Using mouse embryonic fibroblasts from $Cbl^{+/+}$ and $Cbl^{-/-}$ mice, we demonstrate that endogenous Cbl mediates the ubiquitination of Fyn and dictates the rate of Fyn turnover. By analyzing CHO-TS20 cells with a temperature-sensitive ubiquitin activating enzyme, we demonstrate that intact cellular ubiquitin machinery is required for Cbl-induced degradation of Fyn. Analyses of Cbl mutants, with mutations in or near the RING finger domain, in 293T cells revealed that the ubiquitin ligase activity of Cbl is essential for Cbl-induced degradation of Fyn by the proteasome pathway. Finally, use of a SRE-luciferase reporter demonstrated that Cbl-dependent negative regulation of Fyn function requires the region of Cbl that mediates the ubiquitin ligase activity. Given the conservation of structure between various Src-family kinases and the ability of Cbl to interact with multiple members of this family, Cbl-dependent ubiquitination could serve a general role to negatively regulate activated Src-family kinases.

Keywords: Tyrosine kinase / ubiquitin / regulation / degradation.

Introduction

Src-family kinases (SFKs) constitute a large family of evolutionarily conserved protein tyrosine kinases (PTKs) that mediate crucial biological functions, including critical roles in tissue and organ development, cell differentiation, adhesion and migration, mitogenesis, and immune responses [1, 2]. The ease with which subtle mutations can render SFKs dominantly oncogenic [2] has also made them an important model for understanding the mechanisms of PTK regulation. All SFKs share a conserved domain structure, consisting of a membrane-anchoring N-terminal myristoylation signal, adjacent SH3 and SH2 domains, a kinase domain, and a tyrosine residue near the C-terminal tail whose phosphorylation by the C-terminal Src kinase (CSK) is required for repression [1]. The crystal structures of Src and Hck proteins, together with a large body of mutational data, have established a general model of SFK repression and have suggested potential mechanisms of activation [3, 4]. Intra-molecular SH3 domain binding to a type II polyproline-like helix within the SH2-kinase linker region together with SH2 domain binding to the phosphotyrosine residue near the C-terminus force the kinase domain into an inactive conformation [3, 4]. Activation signals are hypothesized to displace the SH2 and SH3 domains from their intra-molecular ligands, promoting the open, active conformation of the kinase domain and concurrently releasing the SH2 and SH3 domains for assembly of signaling complexes. Consistent with this model, inactivating point mutations in the SFK SH3 or SH2 domains can significantly enhance the kinase activity [5]. Furthermore, mutations within the SH2-kinase linker that abolish its binding to the SH3 domain, or overexpression of high affinity SH3 domain-binding ligands, result in increased kinase activity of Hck, Src, or Lck [6–8]. Similarly, deletion or substitution of the negative regulatory tyrosine within the carboxyl tail of SFKs results in enhanced kinase activity and oncogenesis [2], and deletion of the CSK gene leads to constitutively activated SFKs [9, 10]. Conversely, substitutions that enhance the affinity of the C-terminal phosphotyrosine motif for the SH2 domain decrease the kinase activity [11].

While the above paradigm elegantly accounts for basal repression and provides a plausible scheme for activation of SFKs, it is not clear at present if and how activated SFKs are returned to their basal repressed conformation. Given recent evidence that SFKs require cellular chaperones, such

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as members of the HSP90 family, for proper folding [12], it is likely that cells utilize additional mechanisms for deactivation of SFKs and, by implication, other PTKs. Without such ancillary mechanisms, activated SFKs could accumulate resulting in deleterious consequences for a cell. Recent studies indicate that the proto-oncoprotein Cbl provides one such mechanism for deactivation of SFKs [13, 14].

Cbl is a member of an evolutionarily conserved family of cytoplasmic proteins that have emerged as negative regulators of PTK signaling [15]. The Cbl homologues in *Caenorhabditis elegans* and *Drosophila* function as negative regulators of epidermal growth factor receptor (EGFR) signaling [16]. Furthermore, genetic ablation of murine Cbl produced hypercellularity and altered development of several organ systems [17, 18], whereas Cbl-b deletion led to hyperproliferation and hyperactivation of immune cells resulting in autoimmunity [19, 20].

Recent studies have demonstrated that Cbl functions as a ubiquitin ligase towards activated receptor tyrosine kinases (RTKs), a modification that facilitates sorting of ligand-activated receptors to lysosomes where they are degraded [21–23]. This proposed mechanism is analogous to the genetically well-characterized, ubiquitin-dependent, lysosomal targeting of yeast membrane receptors [24]. It is thought that lysosomal enzymes degrade the extracellular regions of growth factor receptors, while the cytoplasmic portion of these receptors may be targeted for proteasomal degradation [21–23]. Notably, transfection studies have shown that Cbl can target the activated pools of non-receptor PTKs such as Syk, ZAP-70 and the SFK Fyn for degradation [13, 25, 26]. However, the role of Cbl ubiquitin ligase function in the negative regulation of these non-receptor PTKs has not been addressed. Importantly, if non-receptor PTKs are indeed targeted for Cbl-dependent ubiquitination, their fate is likely to differ from that of ubiquitinated RTKs, as their ubiquitination is likely to target them directly to the proteasome rather than serving as a lysosomal sorting signal.

Defining the role of ubiquitination in Cbl-dependent regulation of SFKs is important not only due to the intrinsic biological significance of SFK regulation, but also because these PTKs interact with Cbl in a manner that is far more complex than the interactions of Cbl with other PTK targets [13]. The evolutionarily conserved N-terminal region tyrosine kinase binding (TKB) domain of Cbl, composed of a four-helical bundle, an EF-hand and an incomplete SH2 domain [27], specifically interacts with negative regulatory phosphorylation sites within Syk/ZAP-70 and EGFR tyrosine kinases, providing a basis for the selective recruitment of Cbl to activated pools of these PTKs [23, 25, 28]. Mutations (in Cbl or its target PTKs) that abrogate Cbl TKB domain interaction with PTKs block Cbl-dependent negative regulation of EGFR, platelet-derived growth factor (PDGFR) and Syk/ZAP-70 PTKs [23, 29, 25, 30, 26]. Furthermore, an intact Cbl RING finger domain, which interacts with E2 ubiquitin conjugating enzymes (UBCs) [31], is also required for ubiquitination and downregulation of the EGFR [21, 32]. Notably, the TKB and RING finger domains, without the C-terminal half of Cbl, are sufficient for the negative regulation of Syk or EGFR, as well as the ubiquitination of EGFR [32, 29, 33].

In contrast to Syk/ZAP-70, which interact with Cbl exclusively *via* its TKB domain, and RTKs, which require a Cbl TKB-mediated interaction for negative regulation, SFK regulation by Cbl is more complex. Previous studies have demonstrated that Cbl-SFK association involves binding between the SFK SH3 domain and the proline-rich sequences in the C-terminal half of Cbl [34]. Furthermore, the SH2 domains of SFKs can interact with phosphopeptide motifs in the C-terminal half of Cbl [35], and an uncharacterized motif in Fyn can interact with the Cbl TKB domain [13]. Consistent with these multiple modes of physical association, a TKB domain mutant of Cbl was fully capable of decreasing the levels and activity of Fyn when analyzed in a 293T cell transfection system; abrogation of Fyn SH3 binding to the proline-rich region of Cbl, in addition to a Cbl TKB mutation, was required to block the effect of Cbl on Fyn [13]. Given these complexities of Cbl-SFK association, and the fact that two of these interactions involve the C-terminal region of Cbl that is dispensable for EGFR and Syk/ZAP-70 regulation, it is critical to determine if Cbl-mediated negative regulation of SFKs indeed involves its activity as a ubiquitin ligase.

Several lines of evidence support the possibility that Cbl-mediated negative regulation of SFKs may be mediated through ubiquitination. We showed that coexpression of Fyn with Cbl resulted in Fyn degradation, and cell lines from $Cbl^{-/-}$ mice showed elevated Fyn levels [13]. Recent studies of other SFKs have revealed them to be targets of ubiquitination [36-39]. For example, Blk was reported to interact with the ubiquitin ligase E6AP and undergo E6AP-dependent ubiquitination and degradation [38]. Similarly, oncogenic *v*-Src as well as *c*-Src, the latter in CSK-deficient fibroblasts, were shown to be ubiquitinated; furthermore, treatment with proteasome inhibitors led to increased protein levels [36, 37]. While the role of the Cbl proteins in the above situations has not been investigated, these findings are consistent with Cbl regulation of SFKs *via* ubiquitination.

Here, we have addressed this hypothesis through analyses of $Cbl^{+/+}$ and $Cbl^{-/-}$ cell lines, Chinese Hamster Ovary (CHO) cells with a temperature-sensitive defect in ubiquitin activating enzyme (E1) and 293T cells co-expressing Cbl and its ubiquitination-deficient mutants. We provide direct evidence that Cbl negatively regulates the SFK Fyn by targeting it for ubiquitination, and that ubiquitination is a critical mechanism to regulate Fyn protein levels and activity. Given the conservation of structure among SFKs, and the ability of Cbl to interact with multiple SFKs, Cbl-dependent ubiquitin-

ation may provide a general mechanism to negatively regulate activated SFKs.

Materials and methods

Cells

293T human embryonic epithelial kidney cells and mouse embryonic fibroblasts (MEFs) from wildtype ($Cbl^{+/+}$) and Cbl knockout ($Cbl^{-/-}$) mice were maintained as previously described [13]. The CHO cell line CHO-TS20, harboring a temperature-sensitive ubiquitin activating enzyme (E1), was maintained as previously described [40].

Antibodies

The following antibodies were used: monoclonal antibody (mAb) 12CA5 (anti-influenza hemagglutinin [HA] epitope tag; IgG2b) [41]; mAb anti-ubiquitin (IgG1, MMS-258R) from *Covance* (Richmond, USA), rabbit polyclonal antibody (pAb) anti-p44/42 MAP kinase (9102) from *New England BioLabs* (Beverly, USA), mAb anti-EGFR (IgG2a, sc-120), pAb anti-Fyn (sc-16) and pAb anti-Cbl (sc-170) from *Santa Cruz Biotechnology* Inc. (Santa Cruz, USA).

Expression plasmids

The HA-ubiquitin, pSRαNeo-CD8-ξ chimera, Cbl and Fyn expression constructs in the pAlterMAX plasmid backbone (*Promega*, Madison, USA) and GFP-Cbl expression constructs in the pCDNA3 vector backbone (*Invitrogen*, Carlsbad, USA) have been previously described [13, 29, 25, 33, 42]. The Cbl RING finger mutant C3AHN was previously referred to as Cbl-C3HC4C5 [33].

Transient Transfections

293T cells were transfected as previously described using the calcium phosphate method. Cell lysates were prepared 48 h post-transfection with Triton X-100 lysis buffer [26] supplemented with 0.1 % sodium dodecyl sulfate (SDS) and 0.1% DOC. TS20 cells were transfected using the *Lipofectamine*™ reagent (*Life Technologies*, Carlsbad, USA), according to the manufacturer's protocol. The cells were cultured at 30°C for 56 h, then either maintained at 30°C (permissive temperature for E1 function) or shifted to 42°C (non-permissive temperature). Cell lysates were prepared in the lysis buffer described above.

Generation of Fyn-overexpressing MEFs

$Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs overexpressing Fyn were established by retrovirus-mediated transfection of $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs. The retroviral construct MSCVpac-Fyn-T was generated by subcloning murine Fyn-T cDNA fragment from pAlterMAX-Fyn into EcoRI digested MSCVpac. Retroviral supernatants were produced and used to infect target cells as described [43]. Bulk transfectant lines were selected in 5 μg/ml puromycin (*Sigma*, St. Louis, USA) and used as such.

Immunoprecipitation, gel electrophoresis and immunoblotting

Immunoprecipitations were performed as described [44]. The immunoprecipitated proteins and total cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidine difluoride (PVDF) membranes (NEN, Boston, USA), and immunoblotted with the indicated antibodies as described [45]. Blots were visualized as described [26]. Photographs were generated by direct scanning of films using a *Hewlett Packard* ScanJet 4c™ scanner, Palo Alto, USA).

Pulse-Chase Analysis of Fyn Protein Turnover

Fyn-overexpressing $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs were grown in 150-mm tissue culture dishes to about 70% confluence, labeled for 1 h at 37°C with 300 μCi/ml EXPRE^{35}S^{35}S labeling mix (NEN), and pulse-chase analysis was performed as previously described [13]. Autoradiography signals were quantified by densitometric analysis of bands using *ScionImage* software (version beta 3b, Frederick, USA).

Luciferase Assay

293T cells were transfected by the calcium phosphate method with a serum response element (SRE)-luciferase reporter construct and the appropriate Cbl and Fyn constructs, as previously described [13]. At 48 h post-transfection, cells were lysed with Cell Culture Lysis Reagent (*Promega*, Madison, USA) and lysate protein concentrations were determined using the *Bradford* assay. Luciferase activity was determined on equal protein aliquots using a Monolight 3010C luminometer (*Analytical Bioluminescence Laboratory* Inc., Sparks, USA and *Luciferin* Reagent (*Promega*).

Results

Severely reduced ubiquitination of Fyn in MEFs derived from Cbl-deficient mice

We have previously demonstrated that Cbl targets the Fyn protein for degradation in a 293T cell overexpression system, and that steady-state levels of Fyn were elevated in $Cbl^{-/-}$ MEF and T cell lines [13]. The latter system provided an opportunity to directly assess if endogenous Cbl controls Fyn ubiquitination. In view of the known difficulties in detecting ubiquitinated proteins such as Fyn [46], we estab-

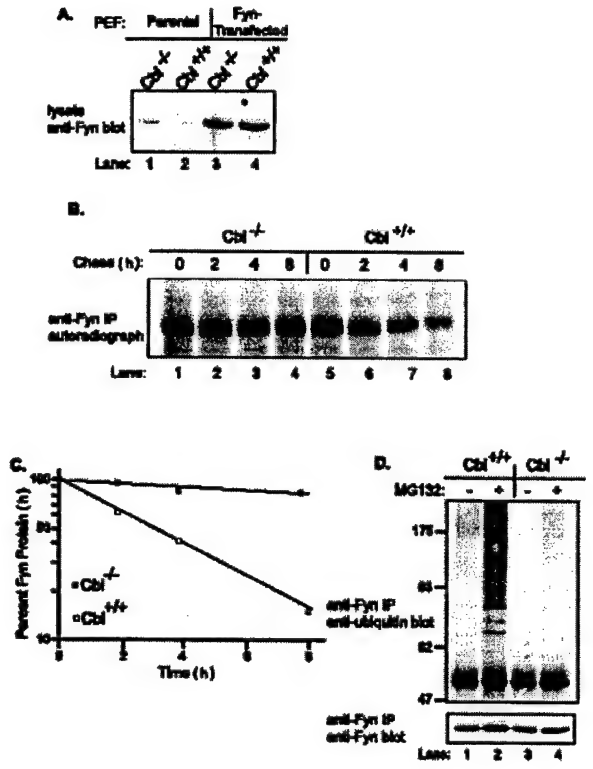

Fig. 1. Stabilization of Fyn protein and impaired Fyn ubiquitination in Cbl$^{-/-}$ primary embryonic fibroblasts.

A. Fyn protein levels in parental *versus* Fyn-transfected primary embryonic fibroblasts (MEFs). Equal amounts (50 μg) of protein lysates from the parental and Fyn-transfected Cbl$^{-/-}$ and Cbl$^{+/+}$ MEFs were resolved by SDS-PAGE and immunoblotted with anti-Fyn antibody.

B. Metabolic pulse-chase analysis of Fyn protein in Fyn-transfected Cbl$^{-/-}$ and Cbl$^{+/+}$ MEFs. Cbl$^{-/-}$ and Cbl$^{+/+}$ MEFs were methionine-starved for 1 h and pulse-labeled with ^{35}S-methionine for 1 h, as described in materials and methods. The cells were incubated in methionine-supplemented, unlabeled medium (chase) for the indicated times (h, hours), and cell lysates were prepared. Anti-Fyn immunoprecipitates (IP) of cell lysates (1 mg) were resolved by SDS-PAGE, and labeled Fyn signals were detected by autoradiography.

C, the radioactive Fyn signals in **B** were quantified using densitometry, expressed as a percentage of the maximal signal intensity and plotted as a function of chase times.

D, impaired Fyn ubiquitination in Cbl$^{-/-}$ MEFs. Fyn-transfected Cbl$^{-/-}$ and Cbl$^{+/+}$ MEFs were incubated with 50 μM MG132 (+) or DMSO control (−) for 5 h and then lysed. Anti-Fyn immunoprecipitates of 1 mg aliquots of lysate were immunoblotted with anti-ubiquitin antibody (top panel), followed by anti-Fyn antibody (bottom panel).

lished a matched pair of Cbl$^{+/+}$ and Cbl$^{-/-}$ MEF lines expressing approximately ten-fold higher levels of Fyn compared to parental MEFs (Fig. 1A).

To determine the impact of the presence or absence of endogenous Cbl on the stability of Fyn protein, we carried out a metabolic pulse-chase analysis of Fyn in the Fyn-transfected Cbl$^{+/+}$ and Cbl$^{-/-}$ MEFs. Equal aliquots of cell lysates were subjected to anti-Fyn immunoprecipitation and radiolabeled Fyn was detected by autoradiography (Fig. 1B). Comparable ^{35}S-Fyn signals were observed in the two cell lines prior to chase (time zero) (Fig. 1B, compare lane 1 with lane 5). Whereas the radiolabeled Fyn signal in Cbl$^{+/+}$ MEFs showed a substantial time-dependent reduction of nearly 80% over the chase period, with a half-life of about 3 h, Fyn protein in Cbl$^{-/-}$ cells was substantially more stable with only a small decrease in signal during the chase period (Fig. 1B and C). These results established that endogenous Cbl controls the stability of the Fyn protein, and provided crucial reagents to directly assess if Cbl regulates the ubiquitination of Fyn.

To assess Fyn ubiquitination in Fyn-transfected Cbl$^{+/+}$ and Cbl$^{-/-}$ MEFs, the cells were incubated for 5 h with (+) or without (−) the proteasome inhibitor MG132, and their lysates were subjected to anti-Fyn immunoprecipitations followed by anti-ubiquitin immunoblotting. A low but detectable ubiquitin signal, seen as a smear similar to ubiquitinated species of other SFKs [37, 39], was observed in anti-Fyn immunoprecipitates of Cbl$^{+/+}$ MEFs incubated without MG132; this signal dramatically increased upon MG132 treatment (Fig. 1D, top panel, compare lane 1 with lane 2). In contrast, the ubiquitin signal was essentially undetectable in anti-Fyn immunoprecipitates of Cbl$^{-/-}$ MEFs and remained very low even after MG132 treatment (Fig. 1D, top panel, compare lane 2 with lane 4). Anti-Fyn immunoblotting showed that MG132 treatment increased the Fyn protein level in Cbl$^{+/+}$ but not Cbl$^{-/-}$ MEFs (Fig. 1D, bottom panel). These findings demonstrate that Fyn protein undergoes ubiquitination, and that the level of endogenous Cbl protein controls the extent of Fyn ubiquitination.

Intact cellular ubiquitination machinery is essential for Cbl-mediated Fyn degradation

Stabilization of Fyn protein in Cbl$^{-/-}$ cells, together with accumulation of ubiquitinated Fyn in MG132-treated Cbl$^{+/+}$ cells, strongly suggested that Cbl-induced ubiquitination serves as a signal for proteasome-mediated degradation. To directly assess the requirement of Fyn ubiquitination for its Cbl-induced degradation, we utilized CHO-TS20 cells. In these cells, the ubiquitin activating enzyme (E1) is fully active at 30°C but nonfunctional at 42°C [40], allowing manipulation of Fyn ubiquitination by using a temperature shift.

Very little Fyn ubiquitination (anti-Fyn immunoprecipitations immunoblotted with anti-ubiquitin) was observed in the ab-

sence of co-transfected Cbl at either temperature (Fig. 2A, lanes 1-5). In contrast, co-expression of Cbl resulted in a marked increase in the levels of ubiquitinated Fyn when cells were maintained at 30°C (Fig. 2A, lane 6). When these cells were shifted to 42°C, Fyn ubiquitination decreased rapidly with nearly undetectable signals after 9 h (Fig. 2A, compare lane 6 with lanes 9-10). Thus, the level of Cbl-induced ubiquitination of Fyn could be precisely regulated in CHO-TS20 cells upon temperature shift.

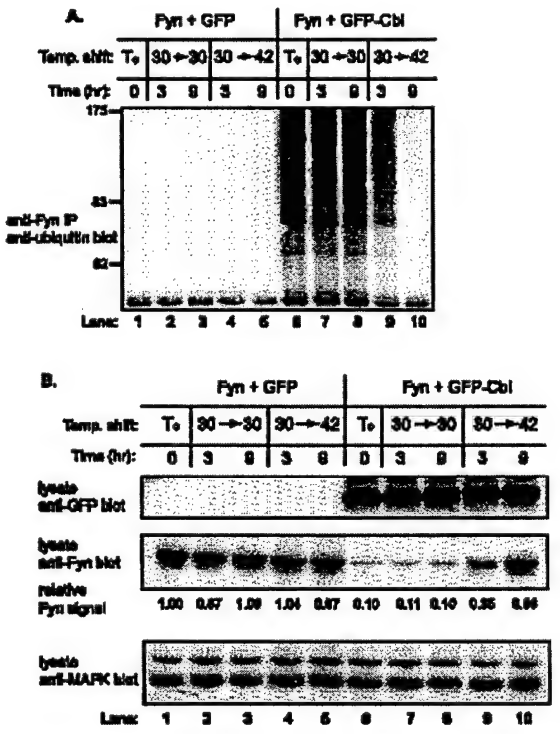

Fig. 2. Cbl-mediated loss of Fyn protein requires intact cellular ubiquitination machinery.

A. impaired Fyn ubiquitination upon E1 inactivation in Cbl-transfected CHO-TS20 cells. CHO-TS20 cells were transfected with Fyn (0.2 μg) expression plasmid together with 4 μg of GFP or GFP-Cbl plasmids and incubated at 30°C for 56 h. At this point (T_o), cells were either maintained at 30°C (30 → 30) or shifted to 42°C (30 → 42) for the indicated times. Anti-Fyn immunoprecipitates from aliquots of lysate protein (1 mg) were immunoblotted with antiubiquitin antibody.

B. stabilization of Fyn protein upon E1 inactivation in Cbl-transfected CHO-TS20 cells. Equal amounts (30 μg) of the same cell lysate used in **A** were immunoblotted with anti-GFP antibody (top panel), anti-Fyn antibody (middle panel), and anti p42/44 MAPK antibody (bottom panel). The levels of Fyn protein were quantified by densitometry, and the values at various times are expressed as a function of the initial Fyn protein level (lane 1) that was assigned a value of 1.0.

The cell lysates used above were directly immunoblotted with anti-GFP and anti-Fyn antibodies to assess the levels of transfected GFP-Cbl and Fyn proteins, respectively. As anticipated, cells co-transfected with GFP-Cbl and Fyn showed a marked reduction in Fyn protein levels when compared to cells cotransfected with GFP vector (Fig. 2B, middle panel, compare lane 1 with lane 6; densitometric units of 1.0 *versus* 0.1). When transfected cells were maintained at the permissive temperature (30°C), no substantial changes in the steady-state levels of Fyn protein were observed. In contrast, when Fyn plus GFP-Cbl transfected cells were shifted to 42°C, a marked time-dependent increase in Fyn protein levels was observed (Fig. 2B, middle panel, lanes 6–10; densitometric units of 0.35 and 0.96 at 3 h and 9 h at 42°C *versus* 0.11 and 0.10 at 30°C, respectively). Relatively little change in Fyn protein level was observed when GFP and Fyn transfected cells were shifted to 42°C (Fig. 2B, middle panel, lanes1–5; densitometric units of 1.04 and 0.97 at 3 h and 9 h at 42°C *versus* 0.87 and 1.09 at 30°C, respectively). Anti-MAP kinase immunoblotting of cell lysates revealed no substantial changes in the levels of MAP kinase protein (Fig. 2B, bottom panel). These results establish that Cbl-mediated degradation of Fyn requires intact cellular ubiquitination machinery.

Cbl-mediated ubiquitination and degradation of Fyn requires an intact Cbl RING finger domain and Fyn SH3 domain

Given the ability of Cbl to control Fyn ubiquitination, we wished to determine if this activity is mediated by the Cbl RING finger-domain encoded ubiquitin ligase activity. For this purpose, we compared the ability of wildtype Cbl protein with its RING finger domain mutants to target Fyn for ubiquitination. To assess Cbl-dependent Fyn ubiquitination *in vivo*, 293T cells were co-transfected with Fyn together with GFP or GFP-Cbl, and a plasmid encoding HA-tagged ubiquitin to facilitate detection of ubiquitinated Fyn.

As expected [32], transfection with GFP-Cbl led to easily detectable ubiquitination of EGFR and this signal was markedly enhanced by MG132 treatment of cells (Fig. 3A, top panel, compare lane 9 with lane 10). Relatively little ubiquitin signal was observed on Fyn in the absence of co-transfected Cbl. In contrast, co-expression of GFP-Cbl led to easily detectable ubiquitination of Fyn, which was accompanied by an expected decrease in the level of Fyn protein (Fig. 3A, compare lane 1 with lane 3). MG132 treatment of cells prior to lysis resulted in marked accumulation of ubiquitinated Fyn and an increase in the level of Fyn protein (Fig. 3A, compare lane 3 with lane 4). Equivalent expression of GFP-tagged

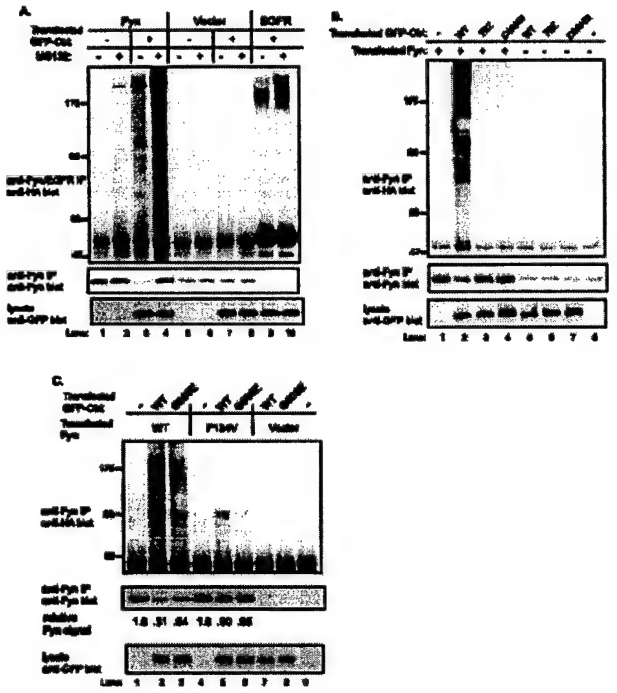

Fig. 3. Cbl-dependent ubiquitination of Fyn in 293T cells and an essential role for the Cbl RING finger domain.

A. Cbl-dependent Fyn ubiquitination is enhanced by treatment with a proteasome inhibitor. 293T cells were transfected with plasmids encoding HA-ubiquitin (7 μg), Fyn (0.15 μg), EGFR (0.15 μg), GFP-Cbl (+) (3 μg) or a GFP (−) control (3 μg). 5 h prior to cell lysate preparation, cells were treated with 50 μM MG132 (+) or DMSO control (−). Anti-Fyn or anti-EGFR immunoprecipitates from aliquots of lysate protein (800 μg) were immunoblotted with anti-HA antibody (top panel) followed by anti-Fyn antibody (middle panel). Equal aliquots (30 μg) of the same cell lysates used above were immunoblotted with anti-GFP antibody (bottom panel).

B. An intact RING finger domain is required for Cbl-dependent Fyn ubiquitination. 293T cells were transfected with the indicated expression plasmids, lysed and anti-Fyn immunoprecipitations were carried out as in **A** and immunoblotted with anti-HA antibody (top panel) and with anti-Fyn antibody (middle panel). The lysate proteins (30 μg) were immunoblotted with anti-GFP antibody (bottom panel).

C. The role of Fyn SH3 domain and Cbl TKB domain-mediated interaction for Cbl-dependent Fyn ubiquitination. 293T cells were transfected with the vector, wildtype Fyn or Fyn-SH3 domain mutant (P134V), lysed and anti-Fyn immunoprecipitations were carried out as in **A** and immunoblotted with anti-HA antibody (top panel) and with anti-Fyn antibody (middle panel). The lysate proteins (10 μg) were immunoblotted with anti-GFP antibody (bottom panel). The levels of Fyn protein were quantified by densitometry, and the values are expressed as a function of the initial Fyn protein level for each Fyn construct (lane 1 and 4) that was assigned a value of 1.0.

Cbl protein in the appropriate lysates was confirmed by anti-GFP immunoblotting of whole cell lysates (Fig. 3A, bottom panel).

Next, we examined if the RING finger domain is required for Cbl-mediated ubiquitination of Fyn. The Cbl mutant C3AHN contains four point mutations predicted to abrogate coordination of both zinc atoms that stabilize the RING finger domain [33], whereas the naturally occurring Cbl-70Z mutant, which is unable to induce Fyn degradation [13], has a deletion of the critical linker region that provides additional essential contacts for UBC binding [31]. In contrast to wildtype Cbl, both 70Z and C3AHN Cbl RING finger mutants were unable to mediate Fyn ubiquitination (Fig. 3B, top panel) despite their equivalent or higher expression levels compared to wildtype Cbl (Fig. 3B, bottom panel). Thus, the RING finger domain-mediated ubiquitin ligase activity of Cbl is necessary for Cbl-dependent ubiquitination of Fyn.

Previous studies have demonstrated that the Fyn SH3 domain provides the primary mode of association with Cbl by interacting with its proline-rich region [13]. Furthermore, mutation of the Cbl TKB domain alone had no significant effect on Cbl-mediated Fyn degradation. However, the Cbl TKB domain was capable of associating with Fyn [13], and recent studies using Src have suggested that the Cbl TKB domain binds to the SFK activation loop phosphorylation site [47], which is conserved among all SFKs. In order to extend our structure-function analyses and establish which domains of Cbl and Fyn are required for Fyn ubiquitination, we tested the ability of a Fyn SH3 domain mutant (P134V) [13] to be ubiquitinated by wildtype Cbl or its TKB domain mutant (G306E). Compared to wildtype Cbl, the TKB domain mutant was still able to mediate Fyn ubiquitination and degradation although to a lesser extent (Fig. 3C, top and middle panel, compare lanes 1–3) when expressed at a level equivalent to wildtype Cbl (Fig. 3C, bottom panel). In contrast, the Fyn SH3 domain mutant showed only minor ubiquitination and degradation when coexpressed with wildtype Cbl. Essentially no ubiquitination or degradation of the Fyn SH3 mutant was observed when coexpressed with the Cbl G306E mutant, and Fyn protein levels were unchanged (Fig. 3C, middle panel). Thus, the Fyn SH3 domain mediates the predominant physical interaction required for Cbl-mediated Fyn ubiquitination, while the Cbl TKB domain appears to mediate a less dominant mode of interaction.

The RING finger domain plays an essential role in Cbl-mediated negative regulation of Fyn-dependent cellular activation

In order to determine the effect of Cbl-dependent ubiquitination on Fyn-mediated cellular activation, we compared the effects of wildtype Cbl and its RING finger domain mutants on Fyn kinase-dependent transactivation of the serum re-

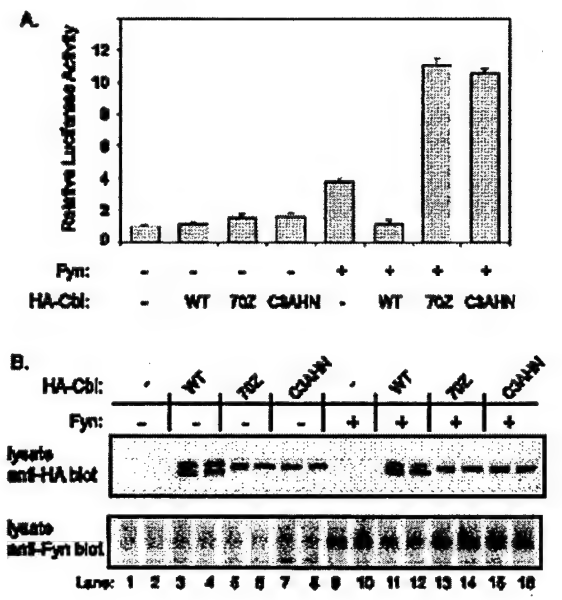

Fig. 4. The RING finger domain is required for Cbl-dependent negative regulation of Fyn-induced transcriptional activation of a SRE-luciferase reporter.

A. mutations in the RING finger domain of Cbl blocks the negative regulation of SRE-luciferase activation. 293T cells were transfected with plasmids encoding the SRE-luciferase reporter (5 μg), CD8-ζ(0.5 μg) and the indicated combinations of Fyn (0.1 μg), HA-Cbl, HA-Cbl-70Z and HA-Cbl-C3AHN (1 μg) or pAlterMAX vector (−). Cells were lysed 48 h after transfection and equal aliquots of lysate protein were used to assay the luciferase activity. The luciferase activity was expressed relative to the activity of lysates transfected with the reporter in the absence of Fyn or Cbl. Results represent the mean +/− one standard deviation of five replicate transfections.

B. analysis of Fyn protein levels in transfected cells used for SRE-luciferase assay. Aliquots of lysate protein (10 μg) from 2 of the 5 replicate samples analyzed in **A** were resolved by SDS-PAGE and immunoblotted with anti-HA (top panel) and anti-Fyn (bottom panel) antibodies.

sponse element (SRE) linked to a luciferase reporter [48]. 293T cells were transfected with the SRE-luciferase reporter plasmid and either Fyn alone or Fyn in combination with wildtype Cbl or its RING finger domain mutants. As expected [13], the expression of Fyn protein led to a modest increase in SRE-luciferase activity compared to mock-transfected cells (Fig. 4A), and this increase was suppressed upon co-expression of wild-type Cbl. In contrast, co-expression of the Cbl RING finger mutant C3AHN as well as the 70Z mutant [13] resulted in a marked enhancement of Fyn-dependent SRE-luciferase reporter activity (Fig. 4A). Expression of Cbl proteins without Fyn had no effect on the SRE luciferase activity. Analysis of cell lysates demonstrated the expected effects of Cbl proteins on Fyn protein levels and confirmed the equivalent expression of various Cbl constructs (Fig. 4B). Overall, these data demonstrate that the RING finger domain, which is required for Fyn ubiquitination and degradation, is also critical for functional negative regulation of Fyn by Cbl.

Discussion

The recently identified function of Cbl as a ubiquitin ligase [21, 32] and our earlier results that Cbl functions as a negative regulator of SFKs [13] led us to hypothesize that Cbl ubiquitin ligase activity provides a physiological mechanism to control the levels of activated SFKs. Here we provide several lines of evidence in support of this hypothesis by examining the regulation of SFK Fyn.

Analyses of multiple cell types, including mouse embryonic fibroblasts, CHO-TS20 cells and 293T human embryonic kidney cells provide evidence for Cbl-dependent ubiquitination of Fyn. An accumulation of ubiquitinated Fyn was also observed upon MG132 treatment of a Jurkat T cell line stably overexpressing Cbl [26] (*Navin Rao, Hamid Band*, unpublished results). Importantly, we show that lack of endogenous Cbl leads to a drastic deficiency in Fyn ubiquitination in $Cbl^{-/-}$ MEFs. The reduction in Fyn ubiquitination in $Cbl^{-/-}$ cells is accompanied by a substantial increase in endogenous Fyn levels [13] and a marked increase in the half-life of the Fyn protein, indicating that Cbl-dependent ubiquitination is a critical determinant of Fyn turnover. It is notable that there are two other mammalian Cbl family members [15]. Whether drastically reduced Fyn ubiquitination in $Cbl^{-/-}$ MEFs reflects a lack of expression of other Cbl family members or a lesser role for these proteins in Fyn ubiquitination will require further investigation.

A complimentary line of evidence for a critical role of Cbl-dependent ubiquitination in regulating Fyn protein levels was provided by analyses of CHO-TS20 cells, which express a thermolabile ubiquitin activating (E1) enzyme. This genetic approach provided further evidence that ubiquitin machinery is essential for Cbl to induce the degradation of Fyn. The results obtained in MEFs and CHO-TS20 cells clearly implicate the ubiquitin ligase activity of Cbl in the negative regulation of Fyn. *In vivo* analysis in 293T cells, using Cbl RING finger domain mutants, established that this indeed was the case. Taken together, our results establish Cbl-dependent ubiquitination as an important mechanism of negative regulation for Fyn, a prototype SFK. Given the ability of Cbl to interact with multiple SFKs and the conservation of structure among members of the SFK family, we propose that Cbl-dependent ubiquitination may provide a general mechanism to negatively regulate activated SFKs. The relatively intense

ubiquitin signal on higher molecular weight species, as compared to Fyn signal, is likely due to multi-ubiquitinated Fyn providing increased numbers of epitopes reactive with anti-ubiquitin antibody. We consider it unlikely that these higher molecular weight species represent a Fyn-associated protein, as lysates were prepared in lysis buffer containing SDS and deoxycholate in order to disrupt protein-protein interactions. Indeed, under such conditions, Fyn-Cbl association was disrupted (data not shown). Moreover, these experiments were performed under optimized conditions to detect Cbl-mediated Fyn ubiquitination and degradation. –

Demonstration of a non-receptor PTK as a target of Cbl-mediated ubiquitination is of considerable significance since all of the targets identified previously are RTKs. Ubiquitin modification of RTKs facilitates sorting to lysosomes for degradation, thus accounting for receptor downregulation. In contrast, ubiquitination of non-receptor PTKs, such as Fyn, is likely to serve as a direct targeting signal for proteasomal degradation, as supported by our results using proteasome inhibitors. While *in vivo* studies and the known direct association between Cbl and SFKs are consistent with Cbl-mediated ubiquitination of SFKs, further analyses using purified SFK, Cbl and ubiquitination enzymes in *in vitro* reconstitution assays will be needed to establish this definitively.

It is likely that Cbl-mediated degradation functions in concert with other mechanisms for deactivation of SFKs, such as the return of activated SFKs to their repressed state through CSK-mediated phosphorylation of the C-terminal tyrosine and potential chaperone-mediated folding into a closed, inactive conformation. The ability of Cbl to target SFKs for ubiquitination and degradation also provides a likely explanation for why Cbl, unlike other SH3 domain ligands such as Sin and HIV NEF [48, 6, 8], does not activate SFKs. This proposal is supported by the ability of ubiquitin ligase-deficient Cbl mutants, such as 70Z and C3AHN, to activate rather than downregulate SFK activity.

The proposed role of ubiquitin in Cbl-mediated SFK regulation is consistent with recent findings that other SFKs, such as v-Src, *c*-Src, Lyn and Blk undergo ubiquitination [36–39]. A recent report published while the present paper was under review indicates that Cbl can indeed function as a ubiquitin ligase towards v-Src and *c*-Src [49]. Interestingly, Blk was shown to interact with and serve as a target of the HECT domain-containing ubiquitin ligase E6AP, which has been previously implicated in ubiquitin-dependent degradation of the nuclear tumor suppressor protein p53 by the human papilloma-virus oncoprotein E6 [50]. Whether E6AP is a physiological ubiquitin ligase for Blk or other SFKs, and whether Cbl and E6AP might work in concert are obvious questions that will require further examination.

A number of observations suggest that Cbl-dependent ubiquitination and degradation primarily target the activated pool of SFKs. The SFK SH3 and SH2 domains, which are primarily responsible for association with Cbl [13], are predicted to be intra-molecularly sequestered in repressed SFKs but available for inter-molecular interactions after activation. The additional interaction between Cbl and Fyn, mediated *via* Cbl's TKB domain, is also likely to involve an activation-dependent autophosphorylation site on Fyn, very likely the activation loop phosphorylation site [47]. Consistent with our proposal, mutation of the Fyn SH3 domain drastically reduced Cbl-mediated Fyn ubiquitination and degradation. However, mutation of the Cbl TKB domain also reduced its ability to induce Fyn ubiquitination and degradation quite significantly. Together, these results suggest that both the Fyn SH3 domain and Cbl-TKB domain-mediated interactions, expected only upon activation of Fyn, contribute to Cbl-dependent Fyn ubiquitination. The more dominant effect of abrogating the Fyn SH3 domain-mediated interaction with Cbl may reflect a requirement for this primary association in order for the secondary Cbl TKB-domain-mediated Cbl-Fyn interaction to occur. Further support for selective regulation of the activated pool of SFKs by Cbl is provided by the observation that the level of autophosphorylated Fyn was markedly increased in $Cbl^{-/-}$ MEFs and T cell lines when compared to their $Cbl^{+/+}$ counterparts [13]. We also show here that wild-type Cbl reduces whereas ubiquitination-defective Cbl mutants increase the Fyn-dependent SRE luciferase reporter activity, a readout of the kinase activity of SFKs. Notably, co-expression of Cbl was also shown to reduce the Src-dependent induction of DNA synthesis in NIH 3T3 cells, and the inhibitory effect of Cbl was abrogated by deletion of the RING finger domain [14]. Finally, ubiquitination of Src and Blk also correlated with their kinase activity [36–38], and CSK-deficient cells were shown to have elevated kinase activity but reduced protein levels of Src, Fyn and Lyn [9, 10]. It will be important to determine if reduction in SFK protein levels in these situations is Cbl-dependent.

While our results support a model that the major function of Cbl is to downregulate the level of activated SFKs by ubiquitin-mediated degradation, other studies have suggested that Cbl transduces signals downstream of SFKs. For example, several SFK-mediated cellular functions, such as integrin-induced macrophage spreading and bone resorption by osteoclasts, were severely reduced when cells were treated with Cbl antisense oligonucleotides [51, 52]. Furthermore, introduction of Cbl into v-abl transformed NIH 3T3 cells restored cell adhesion [43, 53], and a truncated Cbl protein (Cbl 1–480) lacking the C-terminal region, enhanced lamellipodia formation in transfected NIH 3T3 cells [54]. It is therefore possible that Cbl can downregulate SFKs by targeting them for ubiquitination while simultaneously serving as an adapter for SH2 domain-containing proteins, thereby positively regulating signal transduction. In this regard, it is notable that the C-terminal phosphorylation sites of Cbl interact with the p85 subunit of PI3 kinase, the Rac/Rho exchange factor Vav and Crk adapter proteins [15], all of which are

known to be involved in cytoskeletal remodeling, cell spreading and cell migration.

In conclusion, our results demonstrate that Cbl functions as a key regulator of the SFK Fyn by enhancing its ubiquitination and subsequent degradation *via* the proteasome. The negative regulatory role of Cbl is dependent on intact cellular ubiquitin machinery as well as the Cbl RING finger domain, which recruits the ubiquitin machinery. Given the ability of Cbl to interact with multiple SFKs and the conservation of structure among various SFKs, we propose that Cbl-dependent ubiquitination may provide a general mechanism to negatively regulate activated SFKs.

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Negative regulation of Lck by Cbl ubiquitin ligase

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The Cbl-family ubiquitin ligases function as negative regulators of activated receptor tyrosine kinases by facilitating their ubiquitination and subsequent targeting to lysosomes. Cbl associates with the lymphoid-restricted nonreceptor tyrosine kinase Lck, but the functional relevance of this interaction remains unknown. Here, we demonstrate that T cell receptor and CD4 coligation on human T cells results in enhanced association between Cbl and Lck, together with Lck ubiquitination and degradation. A $Cbl^{-/-}$ T cell line showed a marked deficiency in Lck ubiquitination and increased levels of kinase-active Lck. Coexpression in 293T cells demonstrated that Lck kinase activity and Cbl ubiquitin ligase activity were essential for Lck ubiquitination and negative regulation of Lck-dependent serum response element-luciferase reporter activity. The Lck SH3 domain was pivotal for Cbl-Lck association and Cbl-mediated Lck degradation, with a smaller role for interactions mediated by the Cbl tyrosine kinase-binding domain. Finally, analysis of a ZAP-70-deficient T cell line revealed that Cbl inhibited Lck-dependent mitogen-activated protein kinase activation, and an intact Cbl RING finger domain was required for this functional effect. Our results demonstrate a direct, ubiquitination-dependent, negative regulatory role of Cbl for Lck in T cells, independent of Cbl-mediated regulation of ZAP-70.

Protein tyrosine kinase (PTK) activation is an early and necessary event for cellular activation upon engagement of antigen receptors such as the B and T cell receptors (TCR) and Fc receptors (1). The initial event involves the activation of membrane-anchored Src-family kinases (SFKs), such as Lck and Fyn, which phosphorylate the immunoreceptor tyrosine-based activation motifs within the signaling subunits of the receptor, thus creating docking sites to recruit Syk or ZAP-70 PTKs. Genetic and biochemical analyses have established that serial SFK and Syk/ZAP-70 activation is required for antigen receptor signaling (1).

Lck plays a particularly important role in the immune system, and this lymphoid-restricted SFK plays a vital role in T cell development and function (2). Although accentuation of the $CD4^-CD8^-$ T cell developmental block in $Lck^{-/-}$ mice by concurrent Fyn-deficiency suggests partial redundancy, Fyn does not restore peripheral T cell activation in $Lck^{-/-}$ mice, thus indicating an essential, nonredundant role of Lck in T cell activation (3–5).

Lck activation by *Herpes saimari* tyrosine kinase-interacting protein (TIP)-transforming protein and mutational analysis of Lck and other SFKs has established that their unregulated activity results in oncogenicity (6, 7). Thus, precise regulation of Lck is vital for physiological function. Intramolecular SH2 domain-binding to the negative regulatory phosphotyrosine residue near the C terminus, and the SH3 domain-binding to the SH2-kinase linker region maintains SFKs in an inactive, closed conformation, accounting for their basal repressed state. On cellular activation, these intramolecular interactions cease resulting in derepression of the kinase domain while concurrently promoting SH2 and SH3 domain-mediated protein–protein interactions that are essential for signal transmission (8).

In contrast to mechanisms of basal repression and activation that are well supported by crystal structural studies (8), mechanisms of SFK inactivation have been less clear. Tyrosine phosphatases, such as SHP-1, provide one likely mechanism (9); however, it is unclear whether dephosphorylation is sufficient to revert activated Lck back into its inactive state, a process that would also require C-terminal Src kinase (CSK)-mediated phosphorylation of Lck and possibly cellular chaperones such as Hsp90 (10). Recent studies indicate that the Cbl protein family provides a new mode of negatively regulating the activated pools of SFKs (11, 12).

With three distinct mammalian members, the Cbl family of multidomain-signaling proteins is highly conserved in sequence and domain architecture from *Caenorhabditis elegans* to man (13, 14). The conserved N-terminal tyrosine kinase-binding (TKB) domain binds to activation-induced phosphotyrosine motifs and the linker helix and a RING finger domain mediate physical interaction with the E2 ubiquitin (Ub) conjugating enzymes of the Ub pathway (15). Thus Cbl can function as an E3 Ub ligase toward activated PTKs bound to the Cbl TKB domain (15). Cbl-mediated ubiquitination of activated receptor tyrosine kinases serves as a lysosomal targeting signal (16), whereas ubiquitination of nonreceptor PTKs Syk and ZAP-70 targets them for proteasomal degradation (17, 18).

Several SFKs, such as Fyn, Src, Lck ,and Lyn, interact with Cbl by way of the SFK SH3 domain binding to the Cbl proline-rich region, and possibly by way of the SFK SH2 domain binding to phosphorylated Cbl (13). Recent results have shown that Cbl can dramatically reduce the pool of active Fyn through enhanced degradation (11), suggesting a role for Cbl in negatively regulating SFKs. However, analyses of Src-dependent cell spreading and migration in macrophages (19) and bone resorption in osteoclasts (20) have suggested a positive role of Cbl in these responses downstream of Src. Therefore, it is unclear whether negative regulation of Fyn by Cbl is a specialized case or generalizable to other SFKs such as Lck. This question is of obvious importance because the vast majority of cellular Lck is anchored to the plasma membrane, whereas Fyn and Src localize primarily to intracellular vesicles (21). Furthermore, only Lck directly associates with T cell coreceptors CD4/CD8. Here, we demonstrate that Cbl and Lck associate upon TCR/CD4 activation and Lck is ubiquitinated and degraded by the proteasome. These studies support a novel role for Cbl-dependent ubiquitination and degradation in the negative regulation of Lck. Together with previous results, using Fyn, this study suggests a general role for Cbl to regulate SFKs.

Materials and Methods

Cells. The 293T human embryonic kidney epithelial cells, T cell lines 230 and 206 from $Cbl^{+/+}$ and $Cbl^{-/-}$ mice, the human $CD4^+$ T cell clone SPF1, and the ZAP-70-deficient Jurkat T cell

Abbreviations: IP, immunoprecipitation; PTK, protein tyrosine kinase; SFK, Src-family kinase; SRE, serum response element; TCR, T cell receptor; TKB, tyrosine kinase binding; Ub, ubiquitin; WT, wild type; HA, hemagglutinin; MAPK, mitogen-activated protein kinase.

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line expressing the SV40 T antigen (p116-T) were all maintained as described (11, 18, 22).

Antibodies. The antibodies used were: monoclonal antibody (mAb) 12CA5 [anti-influenza hemagglutinin (HA)]; mAb 4G10 (anti-pTyr); mAb SPV-T3b (anti-CD3ε); mAb OKT4 (anti-CD4); mAb W6/32 (anti-MHC I); mAb anti-Ub from Covance; rabbit polyclonal antibody anti-p44/42 mitogen-activated protein kinase (MAPK), polyclonal antibody anti-p44/42 phospho-MAPK, and polyclonal antibody anti-phospho-Src from Cell Signaling Technology (Beverly, MA) and mAb anti-Lck and polyclonal antibody anti-Cbl from Santa Cruz Biotechnology.

Expression Plasmids. The Cbl expression constructs in pAlterMAX and pCDNA3 vector backbone have been described (11, 17, 23). To generate pAlterMAX-Lck constructs, pDKCR Lck and mutant constructs (18) were used as templates for PCR followed by cloning into the pAlterMAX vector. The Lck SH2 (R154K), SH3 (W97A), and double mutant were generated by using Quickchange Mutagenesis (Invitrogen). The plasmid encoding HA-Ub was kindly provided by D. Bohmann (European Molecular Biology Organization, Heidelberg, Germany).

Cell Lysis. Cell lysates were prepared in one of the following buffers as indicated in the figure legends: Triton lysis buffer (11); RIPA buffer (0.15 M NaCl/0.05 M Tris, pH 7.5/1% Triton X-100/1% sodium deoxycholate/0.1% SDS), Triton lysis buffer containing 0.1% SDS and 0.5% deoxycholate; and SDS lysis buffer, Triton lysis buffer containing 1% SDS.

Transient Expression. The 293T cells were transfected by using the calcium phosphate method, and p116T cells were transfected by electroporation (11). Cell lysates were prepared 48 h posttransfection.

T Cell Stimulation. The p116-T cells were stimulated through the TCR by adding SPV-T3b antibody for the indicated times, and cells were lysed. SPF1 T cells were washed in RPMI medium 1640 containing Hepes and glutamine, resuspended at 50×10^6/ml, and incubated with the following mAb (ascites 1:100) SPV-T3b, OKT4, or W6/32 (anti-MHC I) control. After incubation on ice for 30 min, the cells were washed, warmed to 37°C, and bound antibodies were cross-linked by using rabbit anti-mouse antibody.

Immunoprecipitation, Gel Electrophoresis, and Immunoblotting. Immunoprecipitations (IPs) were performed as described (17). The immunoprecipitated proteins and total cell lysates were resolved by SDS/PAGE, transferred to poly(vinylidene difluoride) membranes (NEN), immunoblotted with the indicated antibodies and visualized as described (17). Band intensity was quantified by densitometry by using SCIONIMAGE3B (www.Scioncorp.com).

Luciferase Assay. The 293T cells were transfected with a serum response element (SRE)-luciferase reporter construct and the appropriate Cbl and Lck constructs by using the calcium phosphate method, and assays were performed as described (11).

Results

Lck Ubiquitination and Association on TCR Stimulation. We asked whether coligation of the TCR and CD4 on a normal $CD4^+$ human T cell clone, SPF1, induced Lck ubiquitination. Although Lck ubiquitination was undetectable on CD3 ligation, similar to controls, CD4 ligation resulted in a low level of Lck ubiquitination seen as distinct higher-molecular-weight species together with a smear (Fig. 1*A Upper*). Notably, CD3/CD4 coligation resulted in easily detectable Lck ubiquitination. Anti-Lck immunoblotting revealed the higher-molecular-weight bands and

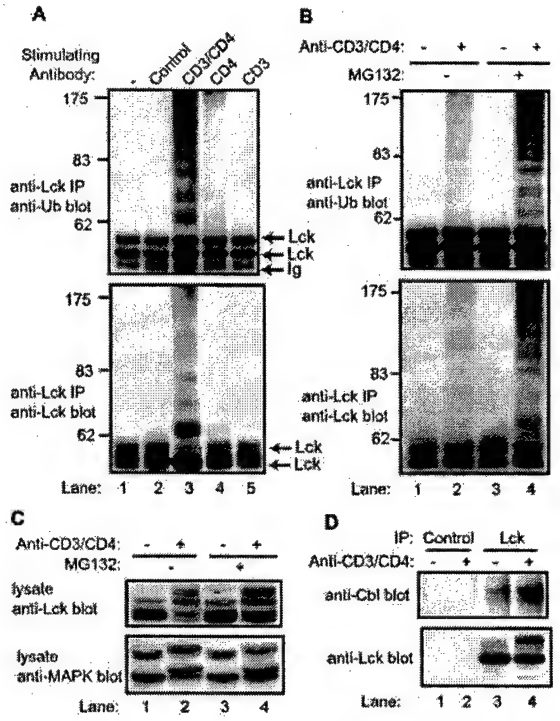

Fig. 1. Lck associates with Cbl and is ubiquitinated upon TCR stimulation in SPF1 T cells. (*A*) Resting $CD4^+$ human SPF1 T cells that had been incubated for 12 h in the absence of IL-2 were stimulated by cross-linking with no antibody (−), control antibody, or anti-CD3/4 antibodies for 10 min at 37°C and then lysed in SDS lysis buffer. Anti-Lck IPs from 1-mg aliquots of lysates was serially probed with an anti-Ub (*Top*) and anti-Lck antibody (*Bottom*). (*B*) SPF1 T cells were treated with 50 μM MG132 (+) or dimethyl sulfoxide vehicle control (−) for 3 h before stimulation for 10 min. IPs were performed and immunoblotted as above. (*C*) Equal amounts of protein lysates from *B* were serially probed with anti-Lck and anti-MAPK antibodies. (*D*) SPF1 T cells were stimulated for 5 min., lysed in Triton lysis buffer, and anti-Lck and isotype matched control IPs were immunoblotted with anti-Cbl (*Top*) or anti-Lck antibody (*Bottom*).

smear to be Lck (Fig. 1*A Lower*, lane 3). The more intense Ub vs. Lck signal on higher-molecular-weight species represents an increased Ub epitope density on multiubiquitinated Lck. Anti-CD3/CD4-induced Lck ubiquitination was also observed by using human lymphoblast preparations freshly derived from peripheral blood (data not shown). The induction of Lck ubiquitination by various stimuli corresponded to their ability to induce early tyrosine phosphorylation events (data not shown).

Pretreatment of SPF1 T cells with the proteasome inhibitor MG132 resulted in a marked enhancement of Lck ubiquitination compared with control (Fig. 1*B Upper*). Concomitantly, MG132 treatment resulted in enhanced detection of the higher-molecular-weight species in an anti-Lck blot (Fig. 1*B Lower*). Anti-Lck immunoblotting of whole cell lysates also revealed that signals corresponding to unmodified Lck species decreased on CD3/4 coligation, apparently reflecting the shift into higher-molecular-weight bands and protein degradation (Fig. 1*C*, lane 2); notably, MG132 treatment led to a slight but reproducible increase in the intensity of this band (Fig. 1*C*, compare lanes 2 and 4). MAPK levels were comparable in the presence and absence of MG132, indicating that changes in Lck protein were specific. Altogether, these findings demonstrate that TCR plus CD4 coligation induces Lck ubiquitination in normal T cells, and that ubiquitination targets Lck to the proteasome.

The Lck SH3 domain binds to Cbl *in vitro*, and the two proteins

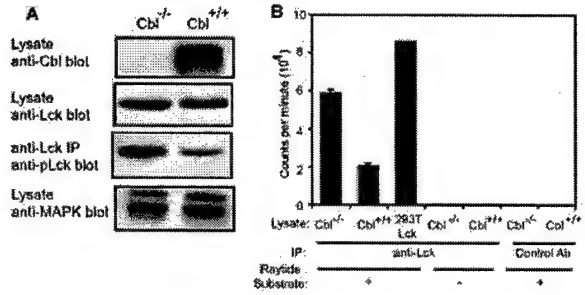

Fig. 2. Cbl$^{-/-}$ T cells have increased levels of kinase-active Lck. (*A*) Cbl$^{-/-}$ and Cbl$^{+/+}$ T cells were lysed in RIPA buffer and equal amounts (50 μg) of protein lysates were immunoblotted with anti-Cbl (top panel), anti-Lck (second panel) and anti-MAPK (bottom panel) antibodies. Anti-Lck IPs from 250 μg of protein lysate was immunoblotted with anti-phospho-Lck (third panel). (*B*) Anti-Lck or isotype matched control IPs from lysates used in *A*, or a positive control lysate from transiently transfected 293T cells, were subjected to *in vitro* kinase assays and the incorporation of ^{32}P signal of [γ^{32}P]ATP into a synthetic Raytide substrate (+) or negative control substrate (−) was quantified. Results are expressed as the mean ± 1 SD of three replicates.

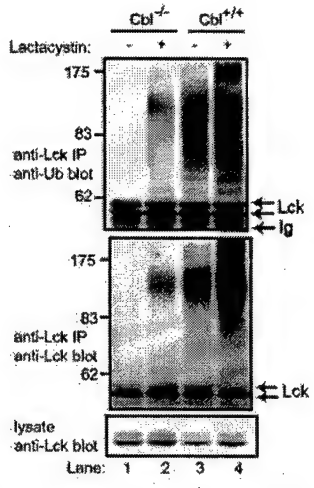

Fig. 3. Impaired Lck ubiquitination in Cbl$^{-/-}$ T cells. Cbl$^{-/-}$ and Cbl$^{+/+}$ T cells were incubated with 10 μM Lactacystin (+) or dimethyl sulfoxide control (−) as well as 0.1 mM orthovanadate for 5 h and then lysed in RIPA buffer. Anti-Lck IPs from 1 mg aliquots of lysate were immunoblotted with anti-Ub antibody (*Top*), followed by anti-Lck antibody (*Middle*). Equal aliquots (30 μg) of cell lysates were immunoblotted with anti-Lck antibody (*Bottom*).

associate *in vivo* (24), suggesting the possibility that Cbl Ub ligase may negatively regulate Lck by means of ubiquitination. In unstimulated SPF1 cells, a low but detectable level of Cbl was coimmunoprecipitated with Lck (Fig. 1*D*, lane 3). Notably, this association substantially increased upon anti-CD3/CD4 stimulation (Fig. 1*D*, compare lanes 3 and 4). Lack of Cbl coimmunoprecipitation in control IPs indicated that the Cbl-Lck association was specific.

Increased Levels of Activated Lck in Cbl$^{-/-}$ T Cells. Given the TCR/CD4-induced Lck ubiquitination and Cbl-Lck association, we asked whether Cbl is required for Lck ubiquitination and degradation by comparing Lck levels in thymocyte-derived, immortalized Cbl$^{-/-}$ and Cbl$^{+/+}$ T cell lines (11). Anti-Cbl immunoblotting of lysates confirmed the expected Cbl deficiency in the Cbl$^{-/-}$ cell line, whereas anti-MAPK immunoblotting showed equivalent sample loading (Fig. 2*A*). Anti-Lck immunoblotting revealed a modestly higher level of total Lck protein in Cbl$^{-/-}$ T cells compared with Cbl$^{+/+}$ cells (Fig. 2*A*, second panel). However, immunoblotting of anti-Lck IPs with an antibody against the phosphorylated activation loop (thus reactive only with activated Lck) revealed a markedly higher level of active Lck in Cbl$^{-/-}$ compared with Cbl$^{+/+}$ cells (Fig. 2*A*, third panel).

To assess directly whether the increased level of autophosphorylated Lck in Cbl$^{-/-}$ cells represented accumulation of kinase-active Lck, anti-Lck IPs were performed with cell lysates (same as Fig. 2*A*) prepared in SDS-containing lysis buffer (to disrupt protein complexes), and subjected to *in vitro* kinase assays. Negligible [^{32}P]ATP incorporation was seen with negative control IPs or if substrate peptide was omitted (Fig. 2*B*). Notably, anti-Lck IPs from Cbl$^{-/-}$ T cell lysates showed 3-fold higher kinase activity than those from Cbl$^{+/+}$ T cells. The accumulation of kinase-active Lck as a result of Cbl deficiency supported a role for Cbl in the ubiquitination and degradation of activated Lck.

Next we asked whether accumulation of Lck in the Cbl$^{-/-}$ T cell line was related to inefficient ubiquitination. A low but detectable Lck Ub signal was observed in Cbl$^{+/+}$ T cells in the absence of the proteasome inhibitor lactacystin; this signal increased markedly on lactacystin treatment (Fig. 3 *Top*, compare lanes 3 and 4). In contrast, the Lck Ub signal was essentially undetectable in Cbl$^{-/-}$ T cells, and the signal remained very low even after lactacystin treatment (Fig. 3 *Top*, compare lanes 1 and 2). The accumulated Lck-Ub could also be visualized with an anti-Lck immunoblot (*Middle*). Furthermore, anti-Lck immunoblotting of whole cell lysate from Cbl$^{+/+}$ cells indicated an accumulation of Lck protein upon lactacystin treatment compared with no change in Cbl$^{-/-}$ cells (*Bottom*). These findings strongly support the conclusion that Lck ubiquitination and protein levels in T cells is controlled by the presence of Cbl protein.

Ubiquitination of Lck in a Reconstitution System. The results in T cells strongly suggested that Lck ubiquitination is a result of its interaction with Cbl. To address this suggestion directly, we compared the ability of the wild-type (WT) Cbl protein vs. the Ub ligase-deficient RING finger mutant C3AHN (23) to target Lck for ubiquitination in transfected 293T cells (Fig. 4*A*). Although relatively little Ub signal was detected on Lck when it was cotransfected with vector (control), coexpression with WT Cbl led to strong Ub signal on Lck, accompanied by a reduction in the level of Lck protein (Fig. 4*A*, compare lanes 1 and 2). In contrast, the Cbl C3AHN mutant was unable to induce Lck ubiquitination or a decrease in Lck protein level (compare lane 2 with lane 3), despite expression at levels comparable with that of WT Cbl (*Bottom*).

Next, we directly tested the role of Lck kinase activity in ubiquitination assays by comparing WT Lck with its kinase active (Y505F) and kinase dead (R273A) mutants (Fig. 4*B*). WT Lck was ubiquitinated and degraded when Cbl was coexpressed, as we had already found. In contrast, constitutively active Lck (Y505F) showed detectable ubiquitination even in the absence of cotransfected Cbl, and this ubiquitination was markedly enhanced when Cbl was coexpressed (compare lane 3 with lane 4). Kinase dead Lck (R273A) was essentially insensitive to Cbl-mediated ubiquitination or degradation (compare lane 6 with lanes 2 and 4). Together, these findings demonstrate that Cbl-mediated ubiquitination of Lck depends on Lck kinase activity and an intact Cbl RING finger domain.

Because Cbl can associate with SFKs through multiple interactions (14), we wanted to determine the relative importance of each interaction for Cbl-mediated degradation of Lck. Coexpression of WT Lck, SH2 mutant (R154K), SH3 mutant

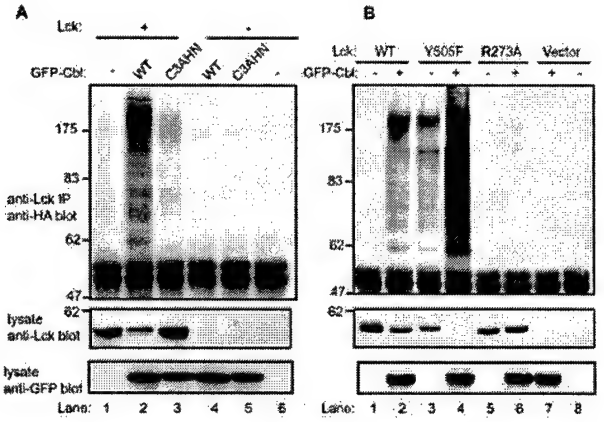

Fig. 4. Cbl-dependent ubiquitination of Lck in 293T cells in dependent on the Cbl RING finger domain and Lck kinase activity. (*A*) 293T cells were transfected with plasmids encoding HA-Ub (5 μg), Lck (0.2 μg), and 3 μg of GFP-Cbl (WT), GFP-Cbl-C3AHN RING finger mutant, or a GFP control (−). Cells were lysed in RIPA buffer, and anti-Lck IPs from 800-μg aliquots of lysate protein were immunoblotted with anti-HA antibody (*Top*). Equal aliquots (30 μg) of cell lysates were immunoblotted with anti-Lck antibody (*Middle*) followed by anti-GFP antibody (*Bottom*). Control GFP is not included in the blot. (*B*) 293T cells were transfected with plasmids encoding HA-Ub (5 μg), Lck (WT), kinase active (Y505F), and kinase dead (R273A) (0.2 μg each), and GFP-Cbl or a GFP control (−) (3 μg). Cells were lysed in RIPA buffer, and immunoblots of anti-Lck IPs were performed as in *A*.

(W97A), or SH3/SH2 double mutant with WT Cbl or the Cbl TKB domain mutant (G306E) followed by coimmunoprecipitation revealed that disruption of the Lck SH3 domain severely abrogated association with Cbl (Fig. 5, compare lane 8 with lane 2). Moreover, disruption of both the SH2 and SH3 domain nearly completely abrogated Lck association with Cbl (compare lane 12 with lane 8). Compared with the SH3 domain mutant, a mutated Lck SH2 domain alone did not significantly disrupt Cbl-Lck association, whereas disruption of the Cbl TKB domain in all

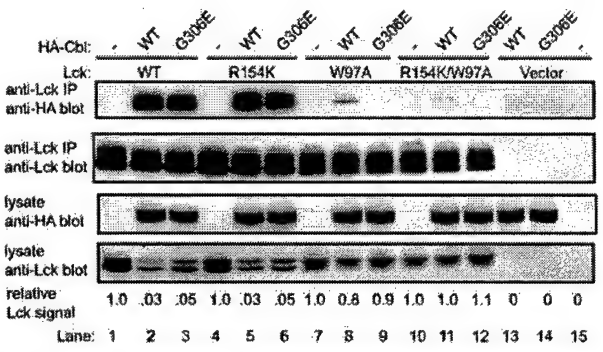

Fig. 5. Relative contribution of Lck SH2 and SH3 domains and the Cbl TKB domain toward Cbl-mediated Lck degradation. 293T cells were transfected with plasmids encoding HA-Cbl (1 μg) and Lck (WT), SH2 (R154K), SH3 (W97A), or double mutants (R154K/W97A) (0.5 μg each). Cells were lysed in Triton lysis buffer and anti-Lck IPs from 1-mg aliquots of lysate protein were immunoblotted with anti-HA antibody (*Top*) and anti-Lck antibody (*Upper Middle*). Aliquots (20 μg) of lysate protein were immunoblotted with anti-HA antibody (*Lower Middle*). For degradation, 293T cells were transfected as above, but with 0.2 μg of the indicated Lck plasmids and lysed with RIPA buffer. 20 μg aliquots of lysate protein were immunoblotted with anti-Lck antibody (*Bottom*). Lck protein levels were quantified by densitometry and are expressed relative to each Lck protein in the absence of coexpressed Cbl.

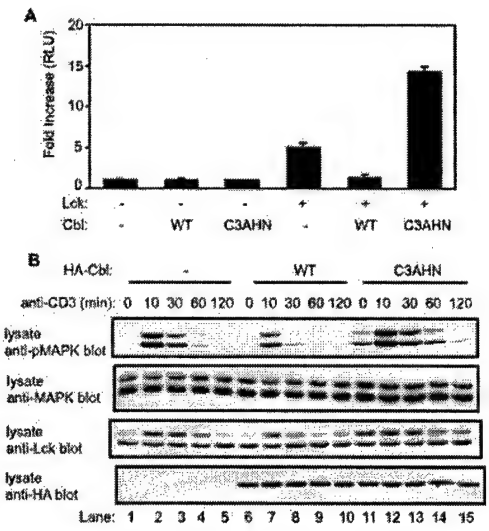

Fig. 6. The RING finger domain is required for Cbl-dependent negative regulation of Lck. (*A*) 293T cells were transfected with plasmids encoding the SRE-luciferase reporter (5 μg) and the indicated combinations of Lck (0.15 μg), HA-Cbl, HA-Cbl-C3AHN and HA-Cbl-70Z (1 μg) or pAlterMAX vector (−). Luciferase activity was expressed relative to activity of lysates transfected with the reporter in the absence of Lck or Cbl. Results represent the mean ± one SD of five replicate transfections. (*B*) Jurkat-derived ZAP-70-deficient p116-T cells, were transfected with 15 μg of plasmid DNA encoding HA-Cbl, HA-Cbl-C3AHN, or pAlterMAX vector (−). Cells were either left unstimulated or stimulated for the indicated times with anti-CD3 antibody before lysis in RIPA buffer. Equal aliquots of cell lysates (25 μg) were subjected to anti-phospho-MAPK (*Top*), anti-MAPK (*Upper Middle*), anti-Lck (*Lower Middle*), and anti-HA (*Bottom*) immunoblotting.

cases slightly decreased Cbl-Lck association. The association data correlated with the ability of Cbl or its TKB domain mutant (G306E) to mediate Lck degradation, as assessed by quantification of Lck protein levels by densitometry (Fig. 5 *Bottom*). Both WT Lck and the SH2 domain mutant were equally sensitive to Cbl-mediated degradation (Fig. 5 *Bottom*, compare lanes 2 and 5). The Lck SH3 mutant was markedly resistant to Cbl-mediated degradation, whereas mutation of both the SH2 and SH3 domains completely blocked degradation (compare lanes 8 and 11). Moreover, the mutation of the Cbl TKB domain slightly blocked degradation of Lck. Together with findings on other SFKs (11, 20), these data suggest that the Lck SH3 domain is the primary mediator of association between Cbl and Lck. The Cbl TKB domain, which specifically binds phosphopeptide motifs (hence phosphorylated Lck), is also involved in this association and subsequent degradation, whereas the Lck SH2 domain plays a much smaller role in Cbl-Lck association and degradation.

The Cbl RING Finger Domain-Dependent Negative Regulation of Lck Function in 293T and Jurkat T Cells. To investigate the functional implications of Cbl-mediated ubiquitination of Lck function, we first compared the effects of WT Cbl and its RING finger domain mutant on Lck kinase-dependent transactivation of the SRE-luciferase reporter (25). Ectopic expression of Lck protein in 293T cells led to a nearly 5-fold increase in SRE-luciferase activity compared with mock-transfected cells (Fig. 6*A*). The Lck-induced increase in SRE-luciferase activity was suppressed to near basal levels upon coexpression of WT Cbl. In contrast, coexpression of the Cbl C3AHN RING finger mutant failed to reduce the Lck-dependent SRE-luciferase reporter activity and, instead, substantially enhanced it (Fig. 6*A*).

Next, we examined the ability of Cbl to regulate Lck function

in a TCR-driven signaling pathway. Studies have established that MAPK activation on TCR cross-linking in Jurkat T cells is Lck-mediated but independent of ZAP-70 (26, 27). Therefore, we used the ZAP-70-deficient Jurkat-derived T cell line, p116, to assess the effect of Cbl on Lck-mediated cellular activation in T cells. This system also avoids any potential effects caused by Cbl-mediated negative regulation of ZAP-70 (18), a downstream target of Lck. As expected (26, 27), MAPK activation was specifically induced by anti-CD3 cross-linking (Fig. 6*B*). Cbl overexpression led to a decrease in the intensity of peak phospho-MAPK signal with a markedly rapid loss of signal over time (30 min vs. 60 min in control). In contrast, although cells transfected with the C3AHN mutant exhibited only slightly enhanced peak phospho-MAPK signal, the signals remained elevated for a considerably longer time (120 min vs. 60 min in control). Furthermore, these results correlated with a decrease of phosphorylated, active Lck (*Upper*) in the presence of Cbl, and enhanced levels of active Lck in the presence of the Cbl RING finger mutant (Fig. 6*B Upper Middle*). Overall, the data in 293T and Jurkat T cell systems demonstrate that Cbl functions as a negative regulator of Lck by its RING finger domain-mediated Ub ligase activity.

Discussion

Lck, a lymphoid-restricted SFK, is essential for T cell development and is indispensable for mature T cell activation (1). The mechanisms that control Lck function are therefore central to regulation of the immune response. The studies presented here demonstrate that Cbl functions as a negative regulator of Lck. We also demonstrate that negative regulation of Lck by Cbl involves ubiquitination and proteasome-mediated degradation of the active pool of Lck and depends on the Ub ligase activity of Cbl. Thus, our studies identify the most proximal T cell PTK as a direct target of negative regulation by Cbl, the prototype of the Cbl family of negative regulators. Recent transfection analyses indicate that Fyn and Src, two ubiquitously expressed SFKs, are also targets of Cbl-induced ubiquitin-dependent degradation (11, 12), raising a strong likelihood that Cbl functions as a general negative regulator of SFKs. Cbl-mediated negative regulation of Lck and other SFKs is likely to provide a regulatory role complementary to other mechanisms, such as CSK-dependent repression and various tyrosine phosphatases.

In contrast to Cbl-mediated ubiquitination of receptor tyrosine kinases, which involves addition of only a few Ub moieties and serves as a sorting signal for transport to lysosomes, the generation of very high-molecular-weight species of SFKs upon ubiquitination indicates that these are multi and/or polyubiquitinated. Such a modification is known to be an efficient proteasomal degradation signal consistent with stabilization of SFKs by proteasome inhibitors (Figs. 1*B* and 3; refs. 28, 29). This distinct nature of SFK ubiquitination as compared with monoubiquitination of receptor tyrosine kinases and other membrane proteins is intriguing given that SFKs in their biologically active forms are myristoylated and/or palmitoylated, and therefore exclusively membrane anchored. Whether this ubiquitination might reflect the localization of SFKs in specialized glycosphingolipid-rich membrane microdomains (30) or the possible involvement of Ub chain elongation machinery are obvious questions that will need to be addressed. Notably, Cbl has been shown to translocate to lipid-rich membranes microdomains upon FcεRI ligation in mast cells (31). It also remains possible that alternate, proteasome-independent mechanisms exist to degrade SFKs.

Our studies also indicate that Cbl-dependent ubiquitination and degradation is specifically directed toward the active pool of Lck. The Cbl-Lck association was markedly induced by TCR/CD4 coligation, consistent with the occlusion of Cbl-binding SH3 and SH2 domains in repressed SFKs. A similar activation-induced association between Cbl and other SFKs has been noted (32, 33). Furthermore, our analyses of $Cbl^{-/-}$ and $Cbl^{+/+}$ cells showed a clear accumulation of the kinase-active pool of Lck (Fig. 2). Finally, an activated mutant of Lck (Y505F) was more susceptible, whereas a kinase dead Lck (D273A) was resistant to Cbl-dependent ubiquitination and degradation compared with WT Cbl (Fig. 4*B*). A similar susceptibility of activated Fyn and Src to negative regulation by Cbl has emerged recently (11, 12). It is likely that the selectivity of Cbl toward activated SFKs reflects the critical role of SFK SH3 domains (Fig. 5) for physical association with Cbl (11), as well as the role of the Cbl TKB domain (Fig. 5) that was observed to bind to the phosphorylated activation loop of Src (20). This motif is fully conserved among most SFKs including Fyn and Lck. Although previous studies have demonstrated that Cbl can bind to the GST-SH2 domain of Lck and Fyn *in vitro* (24, 34), our data indicated a minimal role of the Lck SH2 domain in Lck-Cbl association under our experimental conditions (Fig. 5). Similar data have emerged with Cbl-Fyn interactions (A.G., N.R., and H.B., unpublished data).

Our demonstration of Lck as a direct target of Cbl-mediated negative regulation suggests a significant role of this interaction during T cell development. Although protein expression data are lacking, Cbl mRNA levels are highest in the thymus (15). Importantly, Lck is pivotal for T cell development, being involved in TCR-β allelic exclusion, thymocyte proliferation and positive selection (35). In this regard, it is notable that the $Cbl^{-/-}$ mice show enhanced positive selection of $CD4^+$ thymocytes (36). Studies with Lck transgenes have demonstrated that increased Lck kinase activity can enhance positive selection (37). It is therefore reasonable to postulate that accumulation of active Lck in $Cbl^{-/-}$ thymocytes may mediate the enhanced positive selection that has been described (36). $Cbl^{-/-}$ mice also exhibit increased cellularity in the thymus as well as peripheral lymphoid organs (36, 38). Our findings suggest that Cbl-mediated down-regulation of Lck, in addition to that of ZAP-70, could play a role in this observed phenotype.

Recent findings have implicated SFK ubiquitination in viral pathogenesis. For example, Winberg *et al.* demonstrated that the latent membrane protein 2A of Epstein–Barr virus enhances the ubiquitination of the SFK Lyn in B cells (28). Furthermore, the human papilloma virus E6 oncogene was shown to directly interact with the B cell-specific SFK Blk and induce its degradation through the HECT domain E3 ligase E6AP (39). At present, the role of Cbl in viral inactivation of SFKs remains unknown.

In conclusion, our results demonstrate that Cbl directly regulates the activated T cell-specific SFK Lck, by enhancing its ubiquitination and targeting it for subsequent degradation by the proteasome. Given the highly conserved structure among Cbl family members, the ability of Cbl to control the level of activated Lck suggest critical roles for Cbl family proteins in T cell development and function. Such roles are likely to be revealed when compound Cbl knockouts, such as $Cbl^{-/-}$/$Cbl\text{-}b^{-/-}$ (which are lethal), are examined in the context of the T cell compartment.

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Manuscript in Preparation

Biochemical basis for the requirement of kinase activity in Cbl-dependent ubiquitinylation and degradation of a tyrosine kinase

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INTRODUCTION

Src-family kinases (SFKs) are members of a large family of evolutionarily conserved protein tyrosine kinases (PTKs), with crucial biological roles in tissue and organ development, cell differentiation, adhesion and migration, mitogenesis, and immune responses (Chow, 1995 #1;Thomas, 1997 #3). Relatively subtle mutations can render SFKs dominantly oncogenic(Thomas, 1997 #3) and their deficiencies, either individually or in concert with additional family members, produce severe developmental and/or adult organ dysfunctions. Thus, understanding the mechanisms of their biological function and regulation is of great biological interest. As the first family of PTKs identified, SFKs have also served as an eminent model to understand the mechanisms of PTK regulation in general.

SFKs exhibit a conserved domain structure: a membrane-anchoring N-terminal myristoylation signal, adjacent SH3 and SH2 domains, a kinase domain, and a tyrosine residue near the C-terminal tail whose phosphorylation by the C-terminal Src kinase (CSK) is crucial to repress the enzymatic activity (Chow, 1995 #1). The crystal structures of the kinase and regulatory domains of several SFKs, together with extensive mutational analyses, have defined the molecular basis of SFK repression, and suggested plausible mechanisms of activation (Sicheri, 1997 #2;Xu, 1997 #59). Intra-molecular SH3 domain binding to a type II polyproline-like helix within the SH2-kinase linker region and SH2 domain binding to the C-terminal phosphotyrosine concurrently forces an inactive conformation within the kinase-active cleft and shields the SH2 and SH3 domains from inter-molecular interactions with signaling intermediates(Sicheri, 1997 #2) (Xu, 1997 #59). Activation signals are thought to promote the release of SH2 and SH3 domains from their intra-molecular ligands, promoting the open, active conformation of the kinase domain and allowing SH2 and SH3 domain-mediated assembly of signaling complexes. Indeed, inactivating point mutations in the SFK SH3 or SH2 domains, and deletion or substitution of the negative regulatory tyrosine, deletion of the CSK gene (which mediates the phosphorylation of the C-terminal tyrosine), mutations in the SH2-kinase linker that abolish its binding to the SH3 domain, or overexpression of high affinity SH3 domain ligands lead to constitutively activated SFKs (Thomas, 1997 #3;Seidel-Dugan, 1992 #4;Briggs, 1997 #5;Imamoto, 1993 #6;Nada, 1993 #7).

Conversely, mutational increase in the affinity of the C-terminal phosphotyrosine motif for the SH2 domain decreased the kinase activity of a SFK (Porter, 2000 #8). Thus, it is evident that multiple mechanisms must exist inside a cell to precisely regulate the activity of SFKs.

It is not clear at present how activated SFKs are returned to their basal repressed conformation. A fundamental mechanism to reverse PTK activation is through the activity of phosphotyrosine phopsphatases. However, given the clear evidence that SFKs require cellular chaperones, such as members of the HSP90 family, for proper folding (Bijlmakers, 2000 #9), it is likely that additional mechanisms for deactivation of SFKs and, by implication, other PTKs exist in cells. Recent studies, by our laboratory and others, indicate that the proto-oncoprotein Cbl provides one such mechanism for deactivation of SFKs (Andoniou, 2000 #10;Broome, 1999 #11).

Cbl is a member of an evolutionarily conserved family of multi-domain adaptor-like proteins that function as ubiquitin ligases towards activated PTK (Levkowitz, 1999 #12;Lill, 2000 #13) (Ettenberg, 1999 #14) . Cbl-dependent ubiquitinylation of RTKs facilitates their sorting to the lysosomes where they are degraded (Haglund, 2002 #15;Duan, 2003 #16). Notably, transfection studies have shown that Cbl can target the activated pools of non-receptor PTKs, such as Syk, ZAP-70 and SFKs for degradation, apparently via the proteasome (Rao, 2001 #17;Lupher, 1998 #18;Rao, 2000 #19) (Andoniou, 2000 #10),(Rao, 2002 #60). Thus, Cbl and ubiquitin-dependent degradation has emerged as a major mechanism for negative regulation of PTK signaling (Levkowitz, 1999 #12;Rao, 2002 #21). Indeed, genetic ablation of murine Cbl led to hypercellularity and altered development of several organ systems (Murphy, 1998 #22;Naramura, 1998 #23), whereas Cbl-b deletion led to hyperproliferation and hyperactivation of immune cells resulting in autoimmunity, a phenotype further accentuated by combined T-cell specific Cbl and Cbl-b deficiency (Thien, 2003 #24;Chiang, 2000 #25) (Krawczyk, 2000 #26). Notably, Cbl homologues in Caenorhabditis elegans and Drosophila have been genetically defined as negative regulators of epidermal growth factor receptor (EGFR) signaling (Thien, 2001 #27).

The molecular basis of how Cbl functions as a ubiquitin ligase selectively towards active pools of PTKs has begun to be elucidated(Levkowitz, 1999 #12) (Joazeiro, 1999 #28) (Lee, 1999 #29). Cbl is composed of: an evolutionarily conserved N-terminal tyrosine kinase binding (TKB) domain, itself composed of a four helical bundle, an EF-hand and an incomplete SH2 domain (Meng, 1999 #30), specifically binds to negative regulatory phosphorylation sites induced by autophosphorylation; such motifs have been clearly delineated in Syk/ZAP-70 PTKs and a number of RTKs, generally conforming to an extended motif N/DXpYXXXP/Φ (Φ, hydrophobic); an adjacent conserved motif called RING finger that interacts with ubiquitin conjugating enzymes (Ubc; E2); a proline-rich region; and a C-terminal region that contains a number of inducible tyrosine phosphorylation sites for interaction with SH2 domain-containing proteins, and a ubiquitin-associates (UBA) domain that can bind to ubiquitin (Zheng, 2000 #31;Levkowitz, 1998 #32;Lupher, 1998 #18) (Lupher, 1997 #34). The TKB domain-mediated docking of Cbl to activated PTKs recruits the Cbl RING finger domain-associated E2, which then mediates PTK ubiquitinylation. This mode of interaction is the sole mechanism for Cbl-mediated ubiquitinylation of Syk/ZAP70(Rao, 2001 #17) (Wang, 2001 #35). In the case of RTKs, such as EGFR, a secondary mechanism of Cbl recruitment is provided by adaptor molecules, such as Grb2; Grb2 SH3 domain binds to the proline-rich region of Cbl, whereas its SH2 domain binds to autophosphorylated EGFR (Fukazawa, 1996 #36).

The nature of molecular interactions that mediate Cbl-dependent ubiquitinylation of SFKs is more complex, and the relative roles of the various intermolecular interactions are less well defined. A prominent mechanism of Cbl/SFK association involves the SFK SH3 domain binding to proline-rich sequences in Cbl (Miyake, 1997 #37;Andoniou, 2000 #10). Furthermore, the SH2 domains of SFKs can interact with phosphopeptide motifs in the C-terminal half of Cbl(Tsygankov, 1996 #38) (Hunter, 1999 #39). Finally, the Cbl TKB domain can directly interact with activated SFKs, apparently by binding to the phosphorylated activation loop (Sanjay, 2001 #40). Consistent with these complex intermolecular interactions, a TKB domain mutant of Cbl was fully capable of inducing the degradation of SFK Fyn; abrogation of Fyn SH3 domain binding to the proline-rich

region of Cbl, in addition to a Cbl TKB mutation, was required to effectively block the effect of Cbl on Fyn (Andoniou, 2000 #10). It is notable that the phosphorylation-dependent Cbl/SFK associations involve the C-terminal region of Cbl, which is dispensable for EGFR and Syk/ZAP-70 regulation. Thus, there is clear need to better define the relative importance of various mechanisms of Cbl/SFK association in SFK-directed ubiquitin ligase activity of Cbl.

Given that Cbl is selectively recruited to active PTKs, and is invariably a substrate of these kinases, the potential role of the kinase activity in controlling Cbl-mediated ubiquitinylation of PTKs has been of great interest. Indeed, kinase-dead mutants of Src (Harris, 1999 #54) were markedly resistant to Cbl-dependent ubiquitinylation and degradation, and kinase inhibitors had a similar effect (Yokouchi, 2001 #42). However, it is unclear whether the requirement of the kinase activity of a target PTK is to promote association with Cbl, to induce a conformation that exposes the target lysine residues or by regulating the activity of Cbl. In this context, SFKs offer an excellent model to assess the role of kinase activity per se versus the induced open conformation as determinants of the susceptibility of PTKs to Cbl-dependent ubiquitinylation and degradation, as well-defined mutations in specific motifs can be introduced to promote an open conformation. Here, we have used this approach in the context of the SFK Fyn.

Fyn is a prototype SFK that plays important physiological roles in conjunction with other widely expressed family members, such as Src and Yes(Thomas, 1997 #3) , as well as more selective roles in myelination, neuronal function and T cell development (Grant, 1992 #43;Kitazawa, 1998 #44;Zamoyska, 2003 #45;Molinero, 2003 #46) . We have shown that coexpression of Fyn with Cbl promotes Fyn ubiquitinylation and degradation resulting in negative regulation of Fyn-dependent cellular activation, and that cells from Cbl-/- mice have elevated total and active Fyn levels(Andoniou, 2000 #10) (Rao, 2002 #60). In addition, Cbl-dependent Fyn degradation requires the ubiquitin ligase activity of Cbl and an intact ubiquitinylation machinery in the cell, as revealed by lack of Cbl-dependent Fyn degradation in Chinese Hamster Ovary cells with a temperature-sensitive defect in ubiquitin activating enzyme (E1) (Rao, 2002 #60). By analyzing a series of defined Fyn mutants, we show that both the kinase activity and open conformation

determine the susceptibility of a target PTK to Cbl, primarily by controlling the level of association with Cbl. Thus, the level of association with Cbl appears to be the major determinant of the selective Cbl-mediated ubiquitinylation and degradation of active PTKs.

Materials and Methods:

Cells

293T human embryonic kidney epithelial cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone), 20 mM HEPES, pH 7.35, 1 mM sodium pyruvate,1 mM non-essential amino-acids, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life technologies, Inc.)

GST Fusion Proteins

GST-Cbl-N (GST-Cbl-TKB Domain) fusion protein, incorporating residues 1-357 of human Cbl, and its binding defective mutant GST Cbl N/G306E (G306E) were purified as described(Lupher, 1996 #47) .

Expression constructs and site-directed mutagenesis

The expression constructs encoding the hemagglutinin (HA)- tagged ubiquitin (HA-Ub) (pAlterMAX backbone) (Promega,Madison,USA), CD8-ζ chimera CD8 extracellular and transmembrane domains fused to T cell receptor ζ chain cytoplasmic tail; (pSRαNeo vector), HA-Cbl (pAlterMAX vector), GFP-Cbl (pCDNA3 vector) and Fyn (pAlterMAX vector) have been previously described (Andoniou, 2000 #10;Lupher, 1998 #18;Ota, 2000 #48;Treier, 1994 #49). Point mutants of Fyn and HA-Cbl (described in figure legends and results) were generated by using Quick-Change Site-Directed Mutagenesis Kit from Stratagene (Catalog # 200518) (La Jolla,CA) and appropriate mutagenic primers (sequences available upon request). All of the mutant constructs were sequence verified.

Antibodies

The antibodies used in this study were: monoclonal antibody (mAb) anti-influenza hemagglutinin (HA) epitope tag (12CA5; IgG2b) from Covance Inc., (Richmond, VA), rabbit polyclonal antibody (pAb) anti-p42/44 MAP kinase from Cell Signaling Technologies (Beverly, MA), and pAb anti-Fyn (sc-16) and anti-Cbl (sc-170) from Santa Cruz Biotechnology Inc.,(Santa Cruz, CA,). Phospho-specific anti-Src pAb (against pY416 Src and cross-reactive with Fyn pY417) was from Cell Signaling Technologies (Beverly, MA). (All the antibodies were purchased from the respective companies).

Transient transfections

293T cells were transfected using the calcium phosphate method, as previously described (Andoniou, 2000 #10). Total amounts of DNA in each experiment were kept constant by supplementing with empty vector DNA (pAlterMAX).The Cell lysates used for association studies were prepared in 0.5% Triton-X-100 (Fluka), 50 mM Tris (pH 7.5), 150 mM NaCl, whereas those used for degradation and ubiquitination studies were more stringent RIPA buffer, supplemented with 0.1% sodium dodecyl sulfate (SDS) and 0.5% deoxycholate (DOC). Lysate protein concentrations were measured by the Bradford assay (Bio-Rad Laboratories, Hercules,Calif) with Bovine Serum Albumin (BSA) as standard (BioRad).

Immunoprecipitation and immunoblotting

Immunoprecipitations (IPs) were carried out using Protein A-Sepharose beads (Amersham-Pharmacia Biotecch.), as described previously (Andoniou, 2000 #10). The IPs and cell lysates were resolved on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Produces) ,and serially incubated with the indicated primary antibodies and horseradish peroxidase (HRP) conjugated protein A or anti-mouse antibodies (Cappel-Oreganon Technika, Durham, NC.). Enhanced chemiluminescence (ECL) signals were recorded using a light sensitive film (NEN Life Sciences Products Inc.). Figures represent direct scans of films using a HP ScanJet 4c scanner (Hewlett Packard, Palo Alto, CA).

Results and Discussion:

Previous analyses with EGFR (Schmidt, 2003 #50) and Syk (Paolini, 2002 #51) have shown that an active kinase domain is required for susceptibility to Cbl-mediated ubiquitinylation and degradation. It is however unclear whether the kinase activity of target PTKs is required for Cbl recruitment, to induce a kinase conformation that is more susceptible to ubiquitination, or by regulating Cbl function. If kinase activity is primarily required to promote association with Cbl, then abrogation of kinase activity should concomitantly reduce Cbl association. On the other hand, if the primary role of kinase activity is to promote ubiquitination through the latter two mechanisms, then loss of kinase activity should not reduce association. To address these possibilities in the context of SFKs, we have examined the structural determinants of the susceptibility of Fyn to Cbl-dependent ubiquitinylation and degradation.

To determine if the kinase activity of Fyn is required for its Cbl-dependent ubiquitinylation and degradation, we first compared wildtype (WT) Fyn with its kinase-dead (KD) mutant Fyn-K296R in transient transfection analyses in 293T cells. To assess the effect of Cbl on Fyn ubiquitinylation, we utilized a GFP-Cbl construct and HA-Ub to detect ubiquitinylation. Anti-GFP immunoblotting of cell lysates demonstrated the expression of GFP-Cbl in transfected cells (Fig. 1A, lower panel). As anticipated (Rao, 2002 #60), anti-HA immunoblotting of anti-Fyn immunoprecipitations (IPs) revealed Cbl-dependent enhancement of WT Fyn ubiquitinylation (Fig. 1A, upper panel, lanes 1 and 2). In contrast, the KD mutant of Fyn showed a markedly reduced level of Cbl-dependent ubiquitinylation; however, a significant level of residual ubiquitinylation was still observed (lanes 3 and 4). To assess the role of Fyn kinase activity in Cbl-dependent degradation of Fyn (seen as a decrease in Fyn protein level in anti-Fyn immunoblotting of cell lysates), we cotransfected Fyn with HA-Cbl constructs. In keeping with reduced Cbl-dependent ubiquitinylation of Fyn-KD, the extent of its degradation was also severely reduced (Fig. 1B, lanes 3 and 4 versus lanes 1 and 2). However, a residual level of Cbl-dependent degradation was reproducibly observed.

Given the substantial but incomplete loss of Cbl-mediated ubiquitinylation and degradation by inactivating the kinase activity of Fyn, we asked whether WT and KD Fyn proteins associate with Cbl in a comparable manner. For this purpose, anti-Fyn IPs of 293T cells cotransfected with HA-tagged Cbl and Fyn (WT or KD) were immunoblotted with an anti-HA antibody. Even though the levels of HA-Cbl were comparable and Fyn-KD levels were slightly higher than those of WT Fyn, the level of HA-Cbl coimmunoprecipitation with Fyn-KD was markedly lower than with WT Fyn. This result suggested that a major factor in reduced susceptibility of kinase-inactive Fyn to Cbl-mediated ubiquitinylation may result from reduced association. The residual association of Fyn-KD, however, allowed Cbl-dependent ubiquitinylation and degradation, suggesting that kinase activity is not essential for ubiquitinylation.

Inactive SFKs are folded into a compact structure with occluded SH2 and SH3 domains and a buried kinase site tyrosine residue, thus making these potential mechanisms of interaction unavailable for Cbl association. Thus, the requirement of the kinase activity of Fyn for its susceptibility to Cbl-dependent ubiquitinylation could be due to the open conformation induced by kinase activation, making the various motifs in Fyn available for interaction with Cbl. We utilized specific mutations in Fyn to further address this issue. One major mechanism for the association of WT Fyn and Cbl is via the Fyn SH3 domain binding to Cbl proline-rich region. Therefore, we engineered a Fyn SH2-kinase linker region mutant, Fyn-P251A, which corresponds to Src P250, to release the SH3 domain from its intramolecular ligand (Superti-Furga, 1995 #52). As anticipated (Gonfloni, 1997 #53), Fyn-P251A mutant exhibited a higher level of autophosphorylation on the activation loop tyrosine, indicating its higher kinase activity (data not shown). Furthermore, this mutant showed a higher level of ubiquitinylation in without cotransfection of Cbl (Fig. 2A, upper panel, lanes 5 and 7). Furthermore, this mutant was substantially more susceptible to Cbl-dependent degradation as demonstrated by a markedly lower protein level in anti-Fyn blot (Fig. 2B, lane 1&2 vs 5 & 6). This behavior is consistent with the predicted open conformation of this protein (Superti-Furga, 1995 #52). Notably, when a kinase-inactivating mutation was introduced in Fyn-P251A, the double mutant showed only marginally higher ubiquitinylation and

degradation compared to WT Fyn and was markedly more resistant to ubiquitinylation and degradation compared to Fyn-P251A mutant with an active kinase domain. Thus, while the linker mutation in the context of an active kinase domain markedly enhanced the Cbl-dependent degradation of Fyn, the same mutation in the context of a kinase-dead Fyn protein had relatively little effect to promote Fyn degradation.

Given the reduced Cbl association with Fyn-KD mutant (Fig.2B lower panel lane 4), we assessed the level of Cbl association with Fyn-P251A and Fyn-KD-P251A mutants compared to WT Fyn. Directly correlating with the level of Cbl-dependent ubiquitinylation and degradation, Fyn-P251A mutant showed a substantially elevated association with Cbl even though this mutant was present at markedly reduced levels due to its Cbl-dependent degradation (Fig. 2B, lower panel, lanes 1&2 vs 5 & 6). In contrast, the level of association of Fyn-KD-P251A mutant with Cbl was markedly lower and barely above that of WT Fyn. Thus, even though the linker mutation is predicted to relieve the SH3 domain from its intermolecular ligand, there was relatively little increase in Cbl association and Cbl-dependent ubiquitinylation and degradation.

Aside from the SH3-linker region interaction, the other major intermolecular interaction to keep SFKs in a repressed state is that between the SH2 domain and the C-terminal phosphotyrosine. Therefore, we also examined the susceptibility of Fyn mutants relieved of this intramolecular interaction to Cbl-dependent ubiquitinylation and degradation. As previously noted (Andoniou, 2000 #10) Fyn-Y528F mutant was substantially more susceptible to Cbl-dependent ubiquitinylation and degradation (Fig. 3A, upper panel,compare lanes 2 and 4). When Fyn-528F was further mutated to be kinase inactive, there was a marked loss of Cbl-dependent ubiquitinylation and degradation ; however, low levels of residual ubiquitinylation and degradation were still observed (Fig. 3A, lane 6). Analysis of Fyn-Cbl association revealed that KD version of Fyn-Y528F mutant was markedly impaired in its association with Cbl (Fig. 3B, lower panel, compare lanes 4 and 6).

Given the inability of KD Fyn mutants separately relieved of the two intramolecular interactions (SH3-linker or SH2-tail) to show any significant increase in susceptibility to Cbl, we examined the Fyn mutants in which both intramolecular interactions have been disabled (Fyn-P251A/Y528). Similar to Fyn-P251A and Fyn-Y528F, the kinase-active Fyn-P251A/Y528F mutant was more susceptible to Cbl-dependent ubiquitinylation (Fig. 3A, lane 8) and degradation (Fig. 3B, second panel, lane 8). The KD version of this mutant showed a marked loss of Cbl-dependent ubiquitinylation (Fig. 3A, lane 10) and degradation (Fig. 3B, second panel lanes 10) . When we compared the level of Cbl coimmunoprecipitation with kinase-active versus KD Fyn-P251A/Y528F, we again observed a marked reduction in the association of Cbl with the KD version (Fig.3B lower panel compare lanes 8 and 10). Thus, the level of association of Fyn proteins with Cbl correlated strongly with their susceptibility to Cbl. Importantly, a Fyn protein completely relieved of its intramolecular interactions (presumably an open conformation) did not become more susceptible to Cbl if it lacked the kinase activity.

As residual association as well as Cbl-dependent ubiquitinylation and degradation was observed in all KD mutants, it was likely that this association was mediated via Fyn SH3 domain binding to proline-rich region of Cbl. Indeed, when we introduced a point mutation (P134V) in Fyn-P251A/Y528F-KD mutant, it showed no association with Cbl (Fig. 3B, lower panel, lanes 10 vs 12), and was completely resistant to Cbl-dependent ubiquitinylation (Fig. 3A, upper panel, lanes 9&10 and 11 &12) and degradation (Fig. 3B, second panel, lane 9&10 and 11&12). The behavior of this mutant further emphasizes the tight correlation between the level of association of Fyn proteins with Cbl and their Cbl-dependent ubiquitinylation and degradation. Additionally, to further confirm that both the kinase active and kinase dead mutants were ubiquitinated and degraded by Cbl (albeit in different magnitude), we compared the Fyn levels with the co-transfected Cbl and it's RING finger mutant 70Z.We have observed accumulation of Fyn protein in the presence of 70Z in all of our mutant except the one (Y528F-P251A-P134V-KD) which did not associate with Cbl (Fig 3C middle and lower panels).

The observations above strongly suggested that the kinase activity of Fyn promotes its susceptibility to Cbl by facilitating Cbl/Fyn association. Kinase-active Fyn can interact with Cbl through three potential binding mechanisms: the SH3 domain of Fyn binding to Cbl proline-rich region (as seen above and known to be an important mode of association); Fyn SH2 domain binding to induced phosphorylation sites within the C-terminal half of Cbl (Andoniou, 2000 #10) (Rao, 2002 #60), (Tsygankov, 1996 #38); and a potential interaction between the Cbl TKB domain and a phosphopeptide motif induced on the active kinase (Andoniou, 2000 #10;Sanjay, 2001 #40). We carried out a series of analyses to determine the relative importance of these modes of binding in determining the susceptibility of Fyn to Cbl.

Given the importance of the Cbl TKB domain-mediated interactions in the functional regulation of other target PTKs, we wished to identify and selectively disrupt the TKB domain-binding motif on Fyn for further functional studies. Consistent with previous observations on Fyn and Src(Andoniou, 2000 #10;Sanjay, 2001 #40), pull-down assays using GST-Cbl-N (incorporating Cbl TKB domain) showed that wildtype Fyn can bind to Cbl TKB domain but not to its binding-defective mutant (Cbl-N-G306E) (Fig. 4A, upper panel lane 2 and 3). The predicted Cbl TKB domain-binding sequence in Fyn motif, based on the consensus motif N/DXpYXXXP/Φ(Meng, 1999 #30) corresponds to Fyn activation loop phosphorylation site (DNEYTARQ) . While Fyn-Y417F mutant (similar to Src-Y416F mutant; (Sanjay, 2001 #40) indeed failed to bind to Cbl TKB domain (Fig.4A upper panel lanes 10-12), this mutant showed no autophosphorylation, consistent with the requirement of Y417 phosphorylation for optimal kinase activity, and could not be employed in functional studies. Similar results were observed with mutations (Y417D or Y417E) engineered to partially mimic the negative charge of phosphorylation (Fig 4A upper panel,lanes 13-15 and 16-18), or alanine mutation of the aspartate residue (D415A) at position -2 relative to phosphotyrosine (found critical in phosphopeptide library screening ;(Lupher, 1997 #34) . Finally, none of a panel of mutations in position +4 relative to pY (also critical for binding based on crystal structure of a phosphopeptide-bound Cbl TKB domain; (Meng, 1999 #30) could abrogate Cbl

TKB domain binding to Fyn; in fact, some mutations (e.g. Q421A, Q421E and Q421S) enhanced binding (Fig. 4B, upper panel lanes 3, 9 and lower panel 7). Immunoblotting with an antibody against the phosphorylated SFK activation loop demonstrated that all mutants of Q421were autophosphorylated (Fig.4C).

In view of our inability to design a point mutant of Fyn with retention of kinase activity but loss of Cbl TKB domain binding, we resorted to an alternate strategy to determine the role of Cbl TKB domain-mediated interaction in Cbl-induced ubiquitinylation and degradation of Fyn. As previously reported (Andoniou, 2000 #10;Rao, 2002 #60), Cbl TKB domain mutation in the context of a full-length Cbl protein has relatively little impact on the degradation of Fyn (Fig. 5A, upper panel lanes 2 vs.3). Notably, when the potential interactions mediated by the C-terminal half of Cbl (via Cbl proline-rich region and induced phosphorylation sites binding to Fyn SH3 and SH2 domains, respectively) were eliminated (in Cbl-1-436), less degradation of Fyn was observed (Fig. 5A upper panel, lane 4); however, a TKB domain mutation of this truncated protein (Cbl-1-436-G306E) eliminated its ability to induce the degradation of Fyn (lane 5). We also carried out a reciprocal analysis by eliminating Fyn SH3 domain-mediated Cbl binding (the major mode of interaction; (Andoniou, 2000 #10), (Rao, 2002 #60) and above results). As expected from the increased kinase activity of an SH3 domain mutant of Fyn (Fyn-P134V) (Rao N.), this mutant was substantially more susceptible to Cbl-dependent degradation (Fig. 5A, lanes 1&2 vs 6&7.). Notably, the TKB domain mutation of full-length Cbl led to a markedly reduced level of degradation of Fyn-P134V (Fig. 5A, lanes 7 vs.8). The residual degradation of this mutant by Cbl-G306E is likely to reflect its interactions with Fyn SH2 domain. Importantly, Cbl-1-436, which lacks the ability to interact with Fyn SH2 and SH3 domains, induced marked degradation of Fyn-P134V mutant (Fig 5A, lane 9); in this case, the TKB domain mutant was essentially without effect on Fyn (lane 10). Comparison of WT versus RING finger domain mutant of Cbl demonstrated that the enhanced degradation of Fyn-P134V mutant was indeed dependent on the ubiquitin ligase activity of Cbl (Fig. 5B). Collectively, the results with truncation mutants of Cbl with or without an intact TKB domain, and wildtype and SH3 mutant Fyn, demonstrate that Fyn SH3 domain binding to

Cbl proline-rich region and Cbl TKB domain binding to autophosphorylated Fyn provide predominant mechanism of association between Cbl and activated Fyn, with Fyn SH2 domain-mediated interaction with phosphorylated Cbl providing an additional mechanism. Notably, our results show that the susceptibility of Fyn to Cbl-dependent degradation tightly correlates with the level of physical association between Cbl and Fyn, and that the predominant role of the kinase activity appears to be to promote Cbl/Fyn association.

Studies presented here with Fyn, together with previous analyses of Src, Syk and EGFR (Harris, 1999 #54;Paolini, 2002 #51;Schmidt, 2003 #50), clearly demonstrate that kinase activity of a target PTK is a major factor in determining its susceptibility to Cbl-dependent ubiquitinylation and degradation. However, the biochemical basis for the requirement of the kinase activity has not been elucidated. Previous studies have suggested that one role of the kinase activity is to enhance the ubiquitin ligase function of Cbl apparently by phosphorylation of specific sites in Cbl (Sanjay, 2001 #40),(Levkowitz, 1999 #12). However, these studies did not rule out an enhancement of Cbl/PTK association under their experimental conditions. Furthermore, one potential phosphorylation site that was initially suggested as such a regulatory site, Cbl Y371 within the critical linker region between the TKB and RING finger domains (Levkowitz, 1999 #12) was subsequently shown by crystal structural analysis to be required for structural integrity of the linker helix interaction with the RING finger domain (Zheng, 2000 #31). Our previous results with EGFR and Syk, and present studies with Fyn further demonstrate that a Cbl protein composed only of TKB and RING finger domains can effectively induce the degradation of an active target PTK. This truncated protein lacks the major tyrosine phosphorylation sites (Y700, Y731 and Y774) and is not significantly phosphorylated (Ota, 2000 #48). Thus, it is unlikely that phosphorylation of Cbl on tyrosine residues underlies the requirement of the target PTK activity. Thus, while some direct regulation of Cbl activity through phosphorylation or physical interaction with the target can not be ruled out with certainty, studies presented here suggest that the level of association rather than functional regulation is a major mechanism by which the kinase activity of target PTK influences susceptibility to Cbl.

Furthermore, our results also make it unlikely that the major factor in the enhanced susceptibility of active PTKs to Cbl is due to their open conformation, which could reveal target lysine residues for ubiquitinylation. This conclusion is based on the use of a number of different Fyn mutants relieved of the intramolecular SH3-linker and/or SH2-tail interactions; such mutations are known to promote an open conformation in Src family kinases (Gonfloni, 1997 #53). Our results show that mutants with an open conformation were relatively efficient in association with Cbl. Either the kinase activity is itself required to ensure an open conformation, or the kinase activity is required to promote association with Cbl. Our results favor the latter explanation.

Thus, our analyses with a prototype SFK lead us to conclude that the major role of kinase activity of Cbl targets is to promote their association with Cbl, thereby facilitating their ubiquitinylation. Given the highly conserved structure of SFKs, and of the structural motifs required for Cbl/SFK association, the mechanisms identified here is likely to extend to SFK in general. As the association of Cbl with a number of other targets, including RTKs as well as non-kinase targets, requires phosphorylated tyrosine residues (Rao, 2002 #57;Sanjay, 2001 #58), the notion that kinase activity is required primarily to promote physical association with Cbl targets may eventually provide a general paradigm for Cbl function.

Figure Legends:

Fig 1: Ubiquitination and degradation of Fyn and it's kinase-dead mutant is mediated by Cbl . *A.* HEK 293T cells were transfected with plasmids encoding HA-ubiquitin (5μg),Fyn (0.15μg),GFP-Cbl(+) (3μg) or GFP (-) control (3μg).Cell lysates were prepared 48h after transfection using RIPA-lysis buffer.750μg aliquots of cell lysates were subjected to immunoprecipitation by using anti Fyn antibody (15μg) and resolved by SDS-PAGE and transferred to a PVDF membrane, followed by anti-HA blot (upper panel). A total cell lysate blot was developed using anti-GFP antibody on resolved cell lysates (50μg). *B.* 293T cells were transfected with pAlterMAX-Fyn constructs and its KD mutant (.15μg), with or without pAlterMAX-HA-Cbl expression vector (1μg) encoding HA-tagged Cbl constructs, in 100-mm culture dishes. Cell lysates were prepared 48h after transfection using 0.5% Triton lysis buffer. Anti-Fyn immunoprecipitation was carried out with 750 μg of cell lysate protein. Immune complexes were resolved by SDS-PAGE (9%), transferred to a PVDF membrane overnight, and immunoblotted with anti-HA (12CA5) anti-body (bottom panel). 50 μg of RIPA lysates protein was resolved by SDS-PAGE (9%) and was also transferred to a PVDF membrane, and immunoblotted with anti Fyn (Rabbit-polyclonal) antibody.(middle panel).The same membrane was stripped and reprobed with anti HA antibody (top panel).

Fig. 2. Ubiquitilation, association and degradation of Fyn and it's mutants by Cbl in transfected 293T cells and differential association of Cbl with Fyn and its mutants. *A.* HEK 293T cells were transfected with plasmids encoding HA-ubiquitin (5μg),Fyn (0.15μg),GFP-Cbl(+) (3μg) or GFP (-) control (3μg).Cell lysates were prepared 48h after transfection using RIPA-lysis buffer.750μg aliquots of cell lysates were subjected to immunoprecipitation by using anti Fyn antibody (15μg) and resolved by SDS-PAGE and transferred to a PVDF membrane, followed by anti-HA blot (upper panel).A total cell lysate blot was developed using anti-GFP antibody on resolved cell lysates (50μg).

B. 293T cells were transfected with pAlterMAX-Fyn constructs and its different mutants (.15μg), with or without pAlterMAX-HA-Cbl expression vector (1μg) encoding HA-tagged Cbl constructs, in 100-mm culture dishes. Cell lysates were prepared 48h after

transfection using 0.5% triton lysis buffer. Anti-Fyn immunoprecipitation was carried out with 750 μg of cell lysate protein. Immune complexes were resolved by SDS-PAGE (9%), transferred to a PVDF membrane overnight, and immunoblotted with anti-HA (12CA5) anti-body (bottom panel). 50 μg of RIPA cell lysate protein was resolved by SDS-PAGE (9%) and was also transferred to a PVDF membrane, and immunoblotted with anti Fyn (Rabbit-polyclonal) antibody.(middle panel).The same membrane was stripped and reprobed with anti HA antibody (top panel).

Fig. 3 Differential ubiquitination, association and degradation of Fyn-Y528F and it's different mutants is mediated by Cbl. ***A.*** 293T cells were transfected with plasmids encoding HA-ubiquitin (5μg), Fyn (0.15μg), GFP-Cbl(+) (3μg) or GFP (-) control (3μg).Cell lysates were prepared 48h after transfection using RIPA-lysis buffer.750μg aliquots of cell lysates were subjected to immunoprecipitation by using anti Fyn antibody (15μg) and resolved by SDS-PAGE and transferred to a PVDF membrane, followed by anti-HA blot (upper panel).A total cell-lysate blot was developed using anti-GFP antibody on resolved proteins. ***B.*** 293T cells were transfected with pAlterMAX-FynY528F constructs and it's different mutants (0.15μg), with or without pAlterMAX-HA-Cbl expression vector (1μg), in 100-mm culture dishes. Cell lysates were prepared 48h after transfection using 0.5% triton lysis buffer. Anti-Fyn immunoprecipitation was carried out using 750 μg of cell lysate protein. Immune-complexes were resolved by SDS-PAGE (9%), transferred to a PVDF membrane overnight, and immunoblotted with anti-HA (12CA5) anti-body (bottom panel). 50 μg of total cell lysates protein was resolved by SDS-PAGE (9%) and transferred to a PVDF membrane, and immunoblotted with anti Fyn (Rabbit-polyclonal) antibody (middle panel).The same membrane was stripped and re-probed with anti HA antibody (top panel). ***C.*** 293T cells were transfected with pAlterMAX-FynY528F constructs and it's different mutants (0.15μg), with or without pAlterMAX-HA-Cbl expression vector (1μg) and RING finger mutant 70Z, in 100-mm culture dishes. Cell lysates were prepared 48h after transfection using 0.5% Triton lysis buffer. Anti-Fyn immunoprecipitation was carried out using 750 μg of cell lysate protein. Immune-complexes were resolved by SDS-PAGE (9%), transferred to a PVDF membrane overnight, and immunoblotted with anti-HA (12CA5) anti-body (bottom panel). 50 μg of RIPA lysates protein was resolved by SDS-PAGE (9%) and

transferred to a PVDF membrane, and immunoblotted with anti Fyn (Rabbit-polyclonal) antibody (middle panel).The same membrane was stripped and re-probed with anti HA antibody (top panel)

Fig: 4. Binding assay on wild type Fyn and its different activation loop mutants with GST-Cbl-N and GST-Cbl-N-G306E. ***A.*** 293T cells were transfected with Fyn and it's different activation loop mutants.1mg aliquot of transfected cell lysates were incubated (2h) with glutathione Sepharose beads (20μg) on which GST or GST fusion proteins of Cbl (Cbl-N and Cbl-N-G306E) were non-covalently immobilized (Lupher M. JBC 1996). After incubation at 4^0C, bound polypeptides were washed six times with cold wash buffer and resolved by SDS-PAGE, transferred to a PVDF membrane and immunoblotted with anti Fyn antibody (Top panel). The same membrane was stripped and re-probed with anti-phosphotyrosine antibody (4G10) (bottom panel). ***B.*** 1mg aliquot of transfected cell lysates were incubated (2h, at 4^0C) with glutathione Sepharose beads (20μg) on which GST or GST fusion proteins of Cbl (Cbl-N and Cbl-N-G306E) as in A. Bound polypeptides were resolved by SDS-PAGE, transferred to a PVDF membrane and immunoblotted with anti Fyn antibody . **C.** Aliquots of transfected cell lysates (750μg) of different Fyn activation loop mutants were subjected to immunoprecipitation using anti Fyn antibody (rabbit polyclonal). Immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membrane and immunoblotted with phospho-specific (pY416 Src) antibody.

Fig:5. The role of Fyn SH3 domain and Cbl TKB domain mediated interactions for Cbl mediated Fyn degradation. ***A.***Wild type Fyn or Fyn-SH3 domain mutant (P134V), were co-transfected with full length Cbl , Δ436 and their TKB mutants in 293T cells. The lysate proteins (50mg) were resolved on SDS-PAGE and transferred to a PVDF membrane, followed by anti-Fyn blot (Upper Panel). The same membrane was stripped and re-probed with anti-HA anti-body to see the level of expression of Cbl constructs (middle panel). The membrane was reprobed with p-MAPK antibody to verify the loading control (lower panel) ***B.*** Fyn SH3-mutant (P134V) was co-transfected with HA-tagged Cbl, G306E, Δ436 and Δ436-G306 E and C3AHN constructs into 293T cells. Cell lysates were prepared using RIPA lysis buffer and resolved in SDS-PAGE, transferred to PVDF membrane followed by anti Fyn blot.

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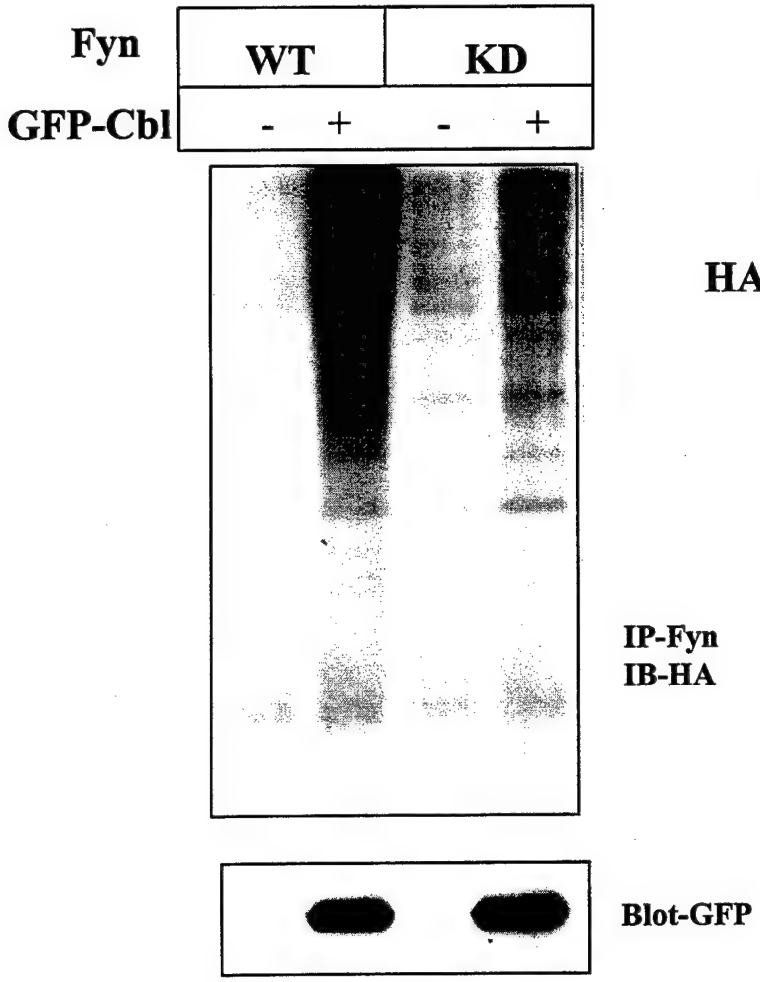
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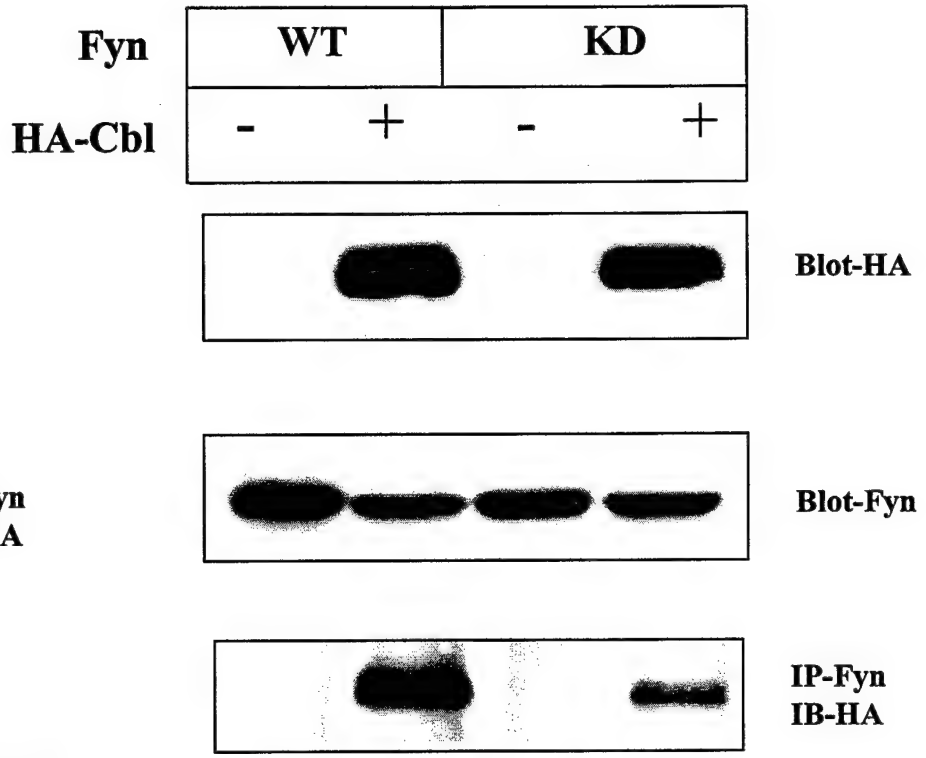
A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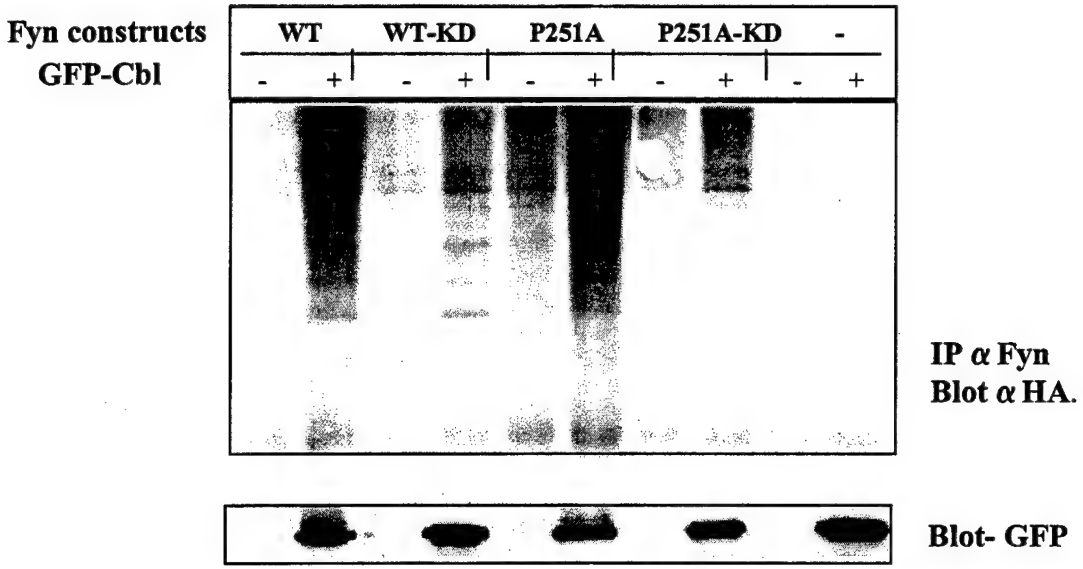
IB-HA

B

Fig:1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- + - + - + - + - +
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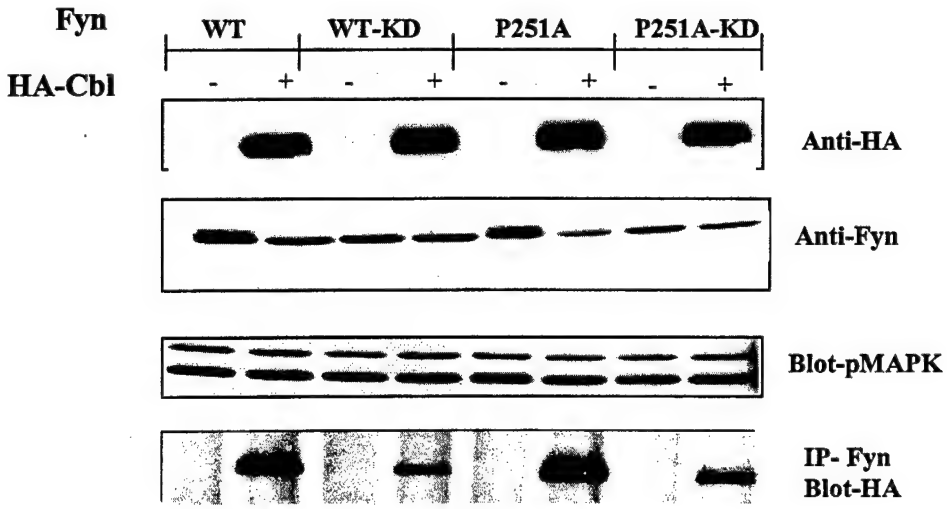
Blot- GFP

Fig: 2A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WT
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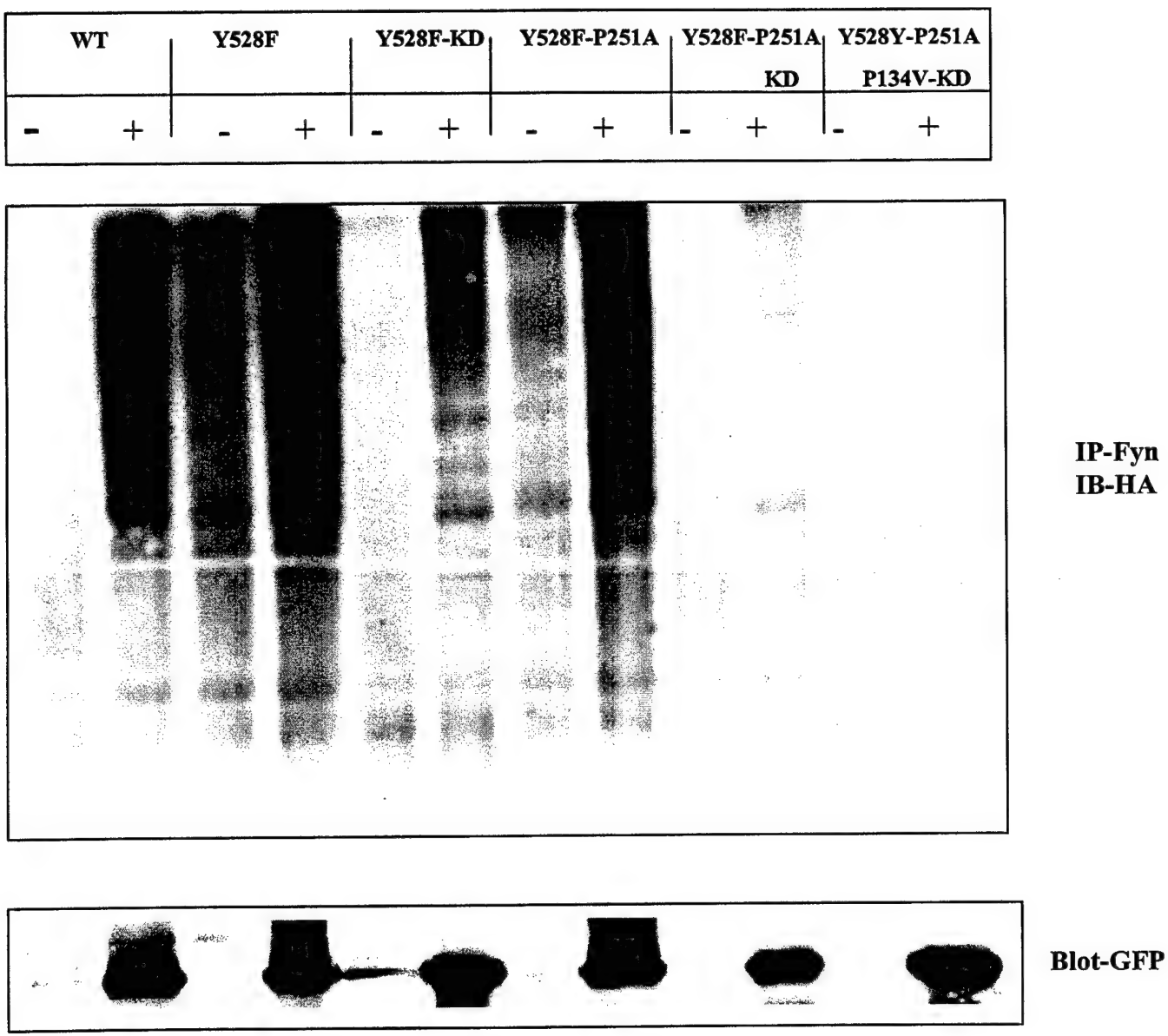
IP- Fyn
Blot-HA

Fig.2B

Fig. 3A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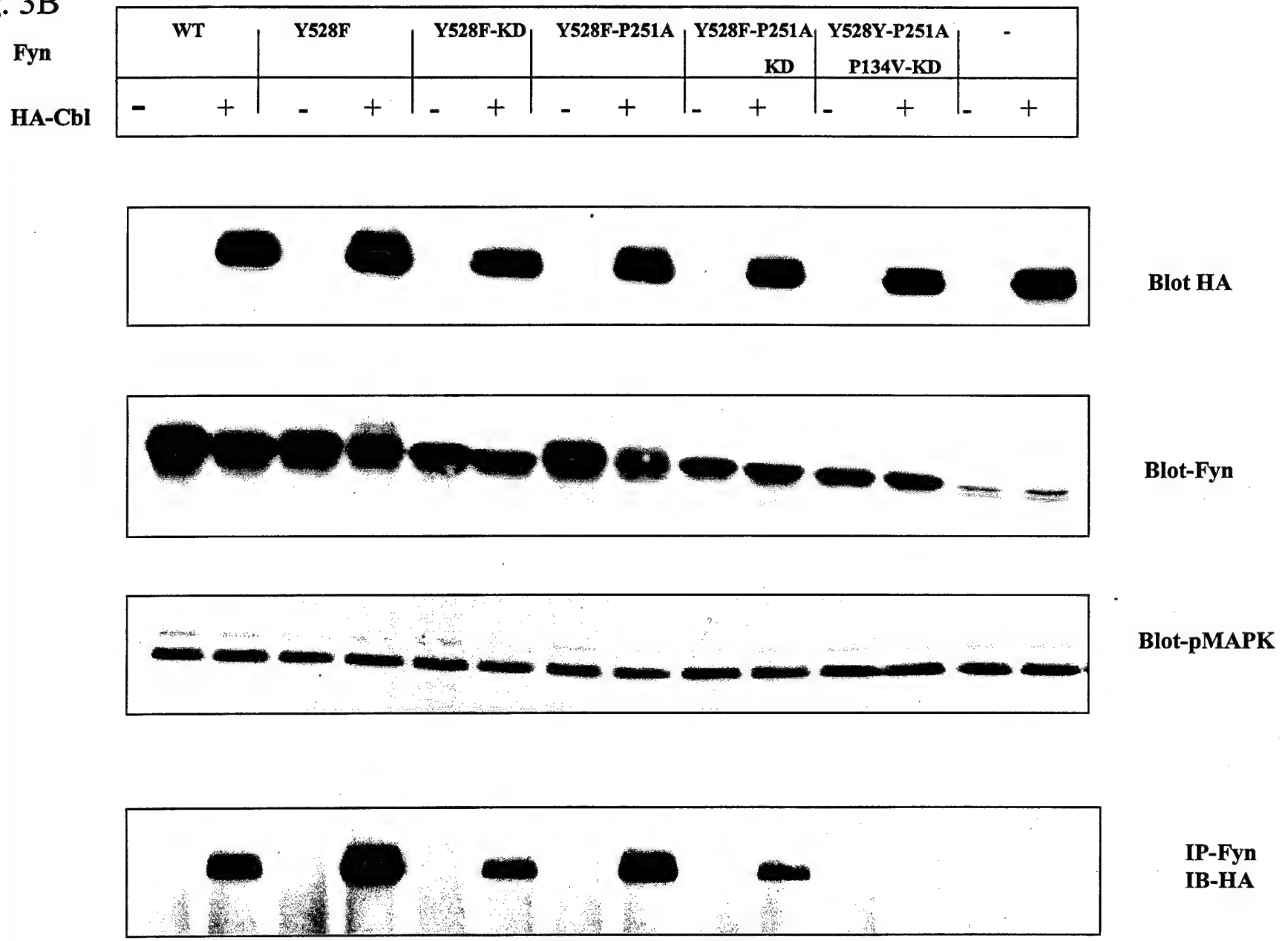
IB-HA
Blot-GFP

Fig. 3B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WT
Y528F
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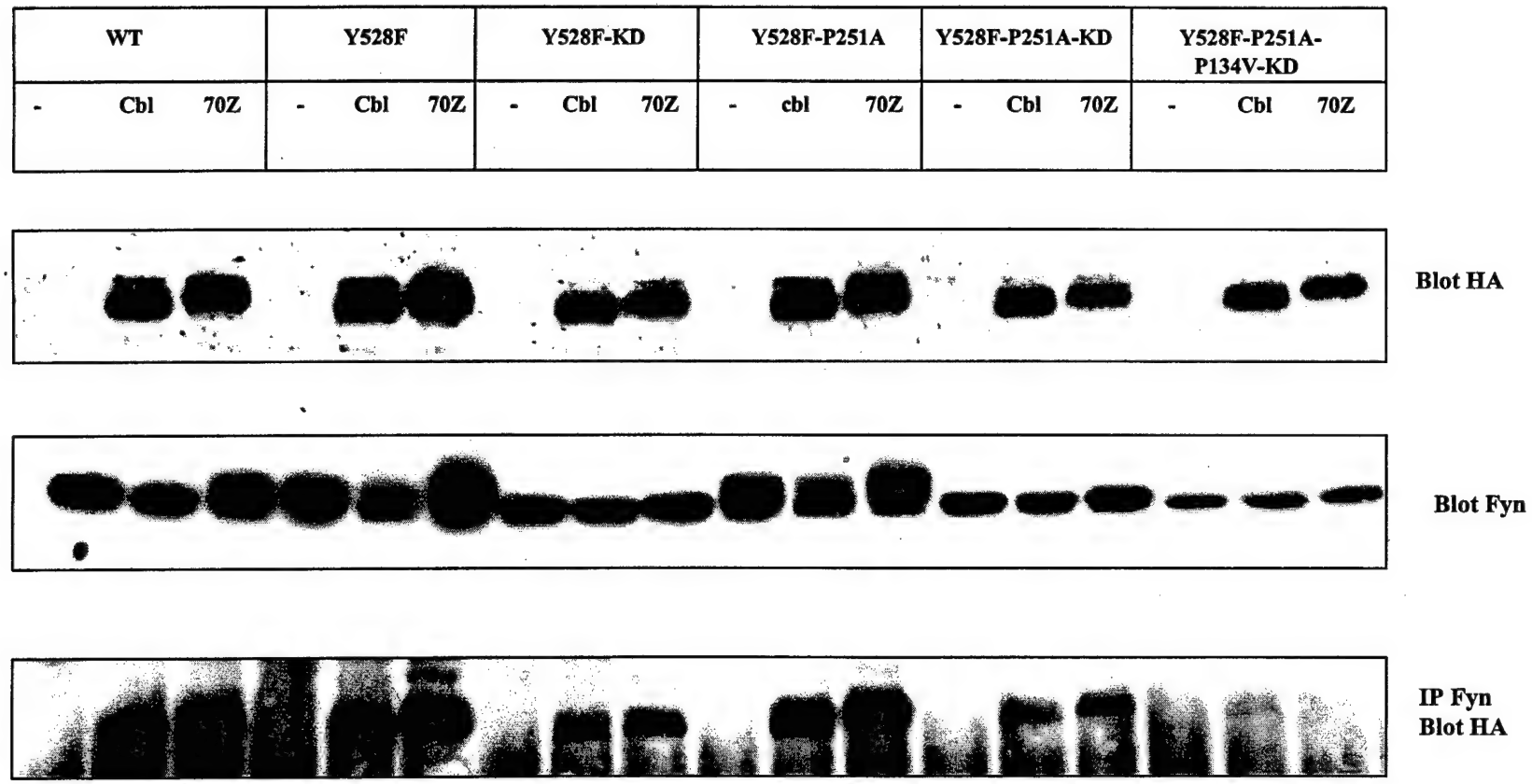
Y528F-P251A KD
Y528Y-P251A P134V-KD
-
HA-Cbl
- + - + - + - + - + - + - +
Blot HA
Blot-Fyn
Blot-pMAPK
IP-Fyn
IB-HA

Fig. 3C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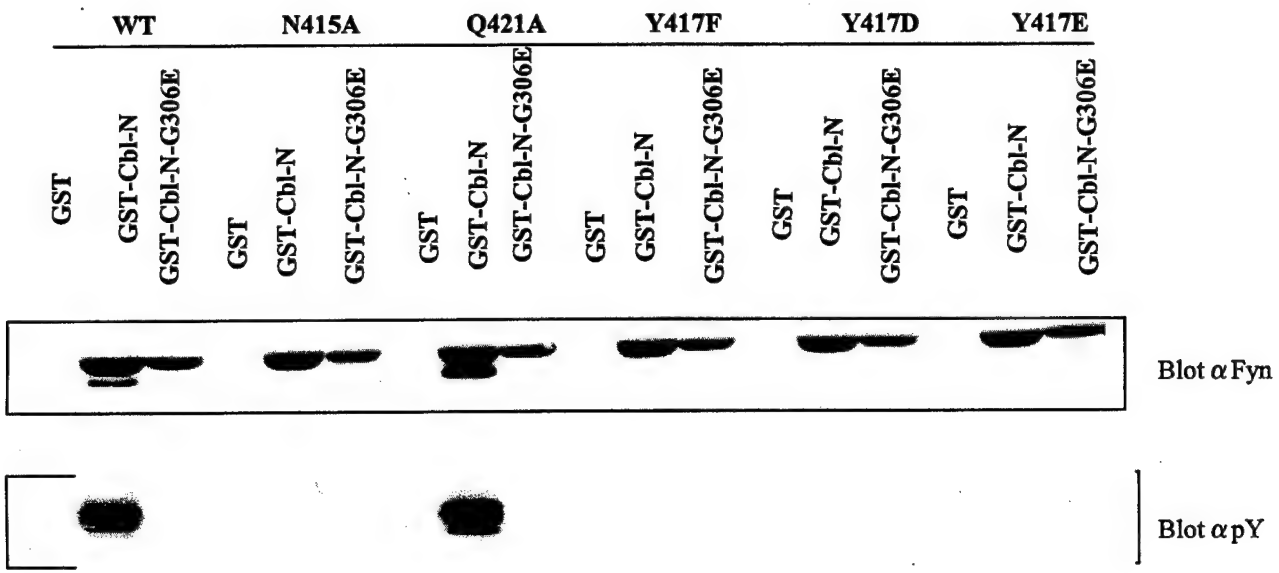
- Cbl 70Z
- cbl 70Z
- Cbl 70Z
- Cbl 70Z
Blot HA
Blot Fyn
IP Fyn
Blot HA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GST-Cbl-N-G306E
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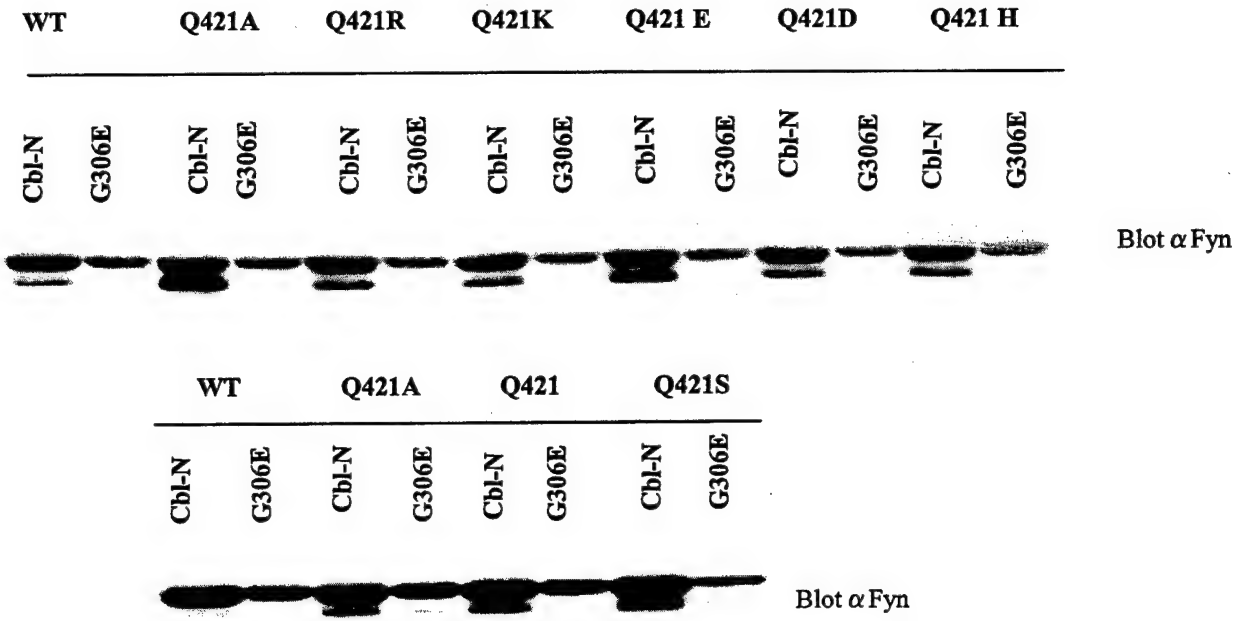
Blot α pY

Fig. 4A.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Q421D
Q421 H
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Q421
Q421S
Cbl-N
G306E
Blot α Fyn

Fig.4B

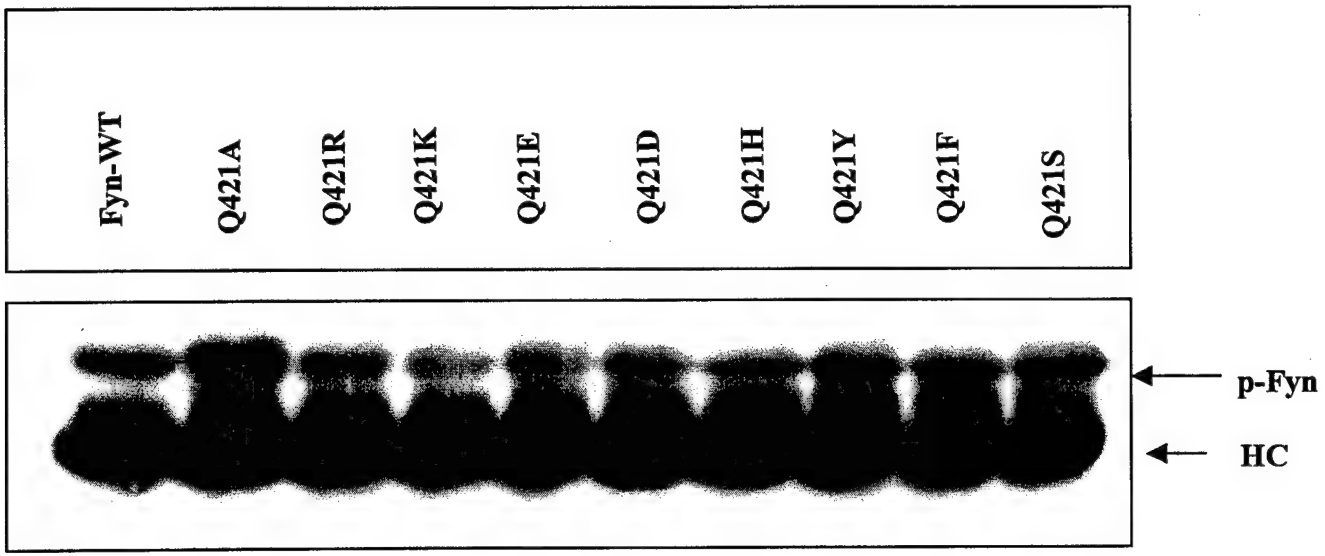
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Fig.4C

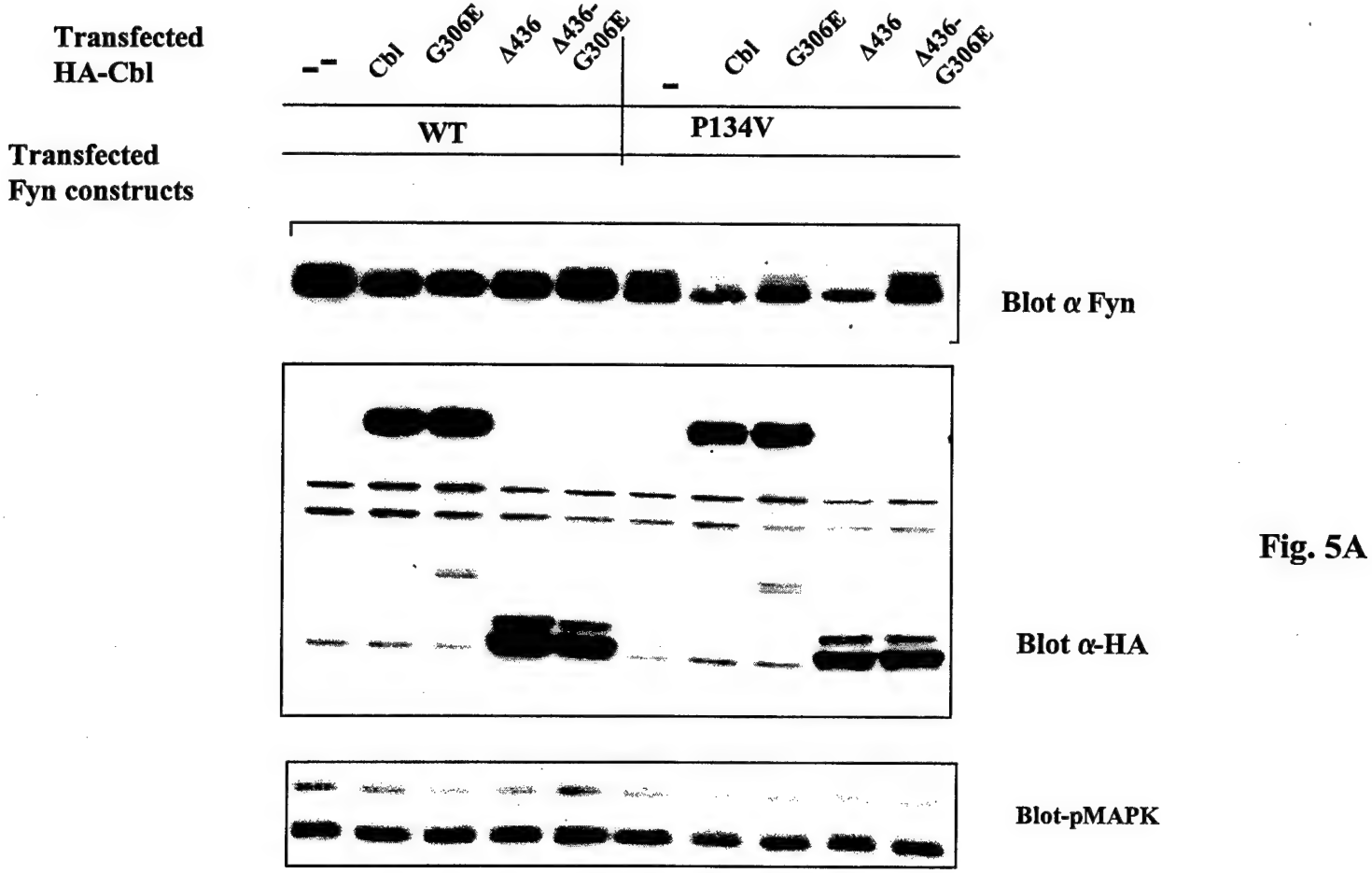
Transfected
HA-Cbl
– Cbl G306E Δ436 Δ436-G306E – Cbl G306E Δ436 Δ436-G306E
WT
P134V
Transfected
Fyn constructs
Blot α Fyn
Blot α-HA
Blot-pMAPK

Fig. 5A

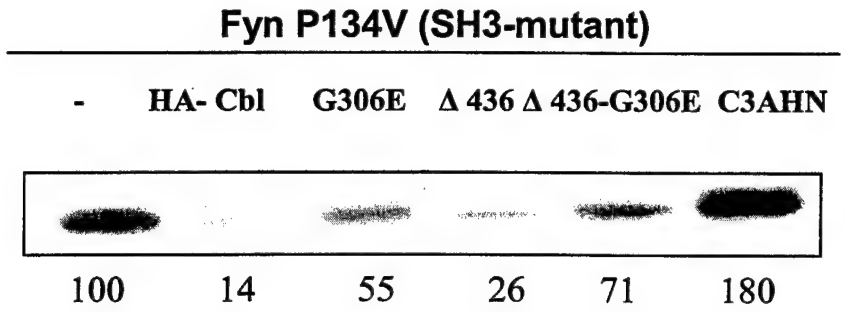
Fyn P134V (SH3-mutant)
- HA- Cbl G306E Δ 436 Δ 436-G306E C3AHN
Blot αFyn
100 14 55 26 71 180

Fig. 5B

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Cbl-mediated ubiquitinylation is required for lysosomal sorting of EGF receptor but is dispensable for endocytosis

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Running Title: Cbl is required for EGFR degradation but not for internalization

Abstract:

Ligand-induced downregulation controls the signaling potency of the EGF receptor (EGFR/ErbB1). Overexpression studies have identified Cbl-mediated ubiquitinylation of EGFR as a mechanism of ligand-induced EGFR downregulation. However, the role of endogenous Cbl in EGFR downregulation and the precise step in the endocytic pathway regulated by Cbl remain unclear. Using $Cbl^{-/-}$ mouse embryonic fibroblast (MEF) cell lines, we demonstrate that endogenous Cbl is essential for ligand-induced ubiquitinylation and efficient degradation of EGFR. Further analyses using CHO cells with a temperature-sensitive defect in ubiquitinylation confirm a crucial role of the ubiquitin machinery in Cbl-mediated EGFR degradation. However, internalization into early endosomes did not require Cbl function or an intact ubiquitin pathway. Confocal immunolocalization studies indicated that Cbl-dependent ubiquitinylation plays a critical role at the early endosome to late endosome/lysosome sorting step of EGFR downregulation. These findings establish Cbl as the major endogenous ubiquitin ligase responsible for EGFR degradation, and show that the critical role of Cbl-mediated ubiquitinylation is at the level of endosomal sorting, rather than at the level of internalization.

Introduction:

Growth factor receptor tyrosine kinases (RTKs) play crucial roles in cellular proliferation, survival, migration and differentiation. EGF receptor (EGFR/ErbB1) is a member of the ErbB family (ErbB1-4) of RTKs, which play crucial homeostatic roles and are implicated in oncogenesis. Ligand-induced activation of RTKs leads to the assembly of signaling protein complexes and subsequent activation of downstream signaling pathways. The ligand-activated RTKs also undergo rapid endocytosis (1). The endocytosed receptors then undergo a sorting process, which determines receptor fate and signal intensity. The receptors can be targeted to the lysosome for degradation, which terminates receptor signals. Alternatively, the internalized receptors can be recycled back to the cell surface for continued ligand binding and signaling (2-5). The relative efficiency of lysosomal sorting versus recycling is a key determinant of the signaling potency of RTKs (6). For example, EGFR is predominantly delivered to lysosomes when activated by EGF. In contrast, heregulin-activated ErbB2 is primarily recycled. The greater efficiency of the recycling process is thought to be a major determinant of the signaling superiority of ErbB2 over EGFR (7-9).

Despite a critical role of endocytic sorting as a determinant of ErbB receptor downregulation, the biochemical mechanisms that regulate this process have only recently begun to be elucidated. We, and others, have identified Cbl as one such regulator (10-12). Cbl is recruited to the activated EGFR through both direct and indirect binding. Direct Cbl-EGFR interaction is mediated through the N-terminal tyrosine kinase-binding (TKB) domain of Cbl, which binds to phosphorylated Y1045 on EGFR (13). Indirect Cbl-EGFR interaction is primarily mediated through Grb2: the SH3

domain of Grb2 binds to proline-rich sequences in Cbl whereas the SH2 domain binds to autophosphorylated EGFR (14). The RING finger domain in Cbl binds to ubiquitin-conjugating enzymes (E2s), allowing Cbl to function as a ubiquitin ligase (E3) towards activated EGFR (15,16). Overexpression studies have demonstrated that Cbl-mediated ubiquitinylation promotes downregulation of EGFR, whereas Cbl proteins with mutations in the RING finger domain, or in a conserved helix connecting the RING finger and TKB domains, reduce the extent of EGFR ubiquitinylation and downregulation (17-19). It has not been established, however, whether endogenous Cbl-mediated ubiquitinylation plays an essential role in EGFR degradation.

Two potential mechanisms for Cbl-mediated, and ubiquitinylation-dependent, EGFR downregulation have been proposed. First, a role for Cbl-mediated ubiquitinylation in EGFR endocytosis has been postulated based on the well-established role of ubiquitinylation in the endocytosis of yeast pheromone receptors (20,21), and the apparent requirement for the ubiquitin pathway in mammalian growth hormone receptor internalization (22). Consistent with this possibility, Cbl was also recently found to associate with a CIN85-endophilin complex and to facilitate the monoubiquitinylation of CIN85 (23). Since a truncated CIN85 mutant that did not associate with Cbl impaired the rate of EGFR internalization, the investigators concluded that Cbl played a role in EGFR endocytosis by serving as an adaptor to link EGFR with CIN85 and endophilin (24). Another study arrived at a similar conclusion in the context of the RTK c-MET (25). More recently, the EGFR Y1068/1086F mutant, which is unable to bind Grb2, was also found to be impaired for endocytosis (26). Interestingly, this mutant was also unable to efficiently recruit Cbl to EGFR, and showed impaired ubiquitinylation. Collectively,

these studies suggest a role for Cbl, and Cbl-regulated ubiquitinylation, in EGFR endocytosis. Other studies, however, argue against a role for Cbl-mediated ubiquitinylation in the initial internalization of EGFR. For example, even though EGFR ubiquitinylation occurs at the cell surface, it did not appear to be required for endocytosis (27,28). In contrast, both ubiquitinylation and proteasome activity were needed for transferring EGFR into internal vesicles of the multi-vesicular body (28). In other studies, dominant-negative Cbl mutants were found to prevent downregulation of EGFR, but EGFR was seen to undergo ligand-induced localization to intracellular vesicular structures (17). Furthermore, the rate of initial internalization did not correlate with the ability of overexpressed Cbl mutants to inhibit EGFR downregulation (18). These results support a role for Cbl and ubiquitinylation at a late step in the endocytic pathway, rather than at the initial internalization step. The relative contribution of these alternate mechanisms in Cbl-mediated downregulation of EGFR has not been clarified.

In the present study, we used CHO cells conditionally defective in ubiquitinylation, and Cbl-deficient mouse embryonic fibroblast (MEF) cells, to address the role of Cbl and Cbl-mediated ubiquitinylation in the internalization, endosomal sorting and degradation of EGFR. We demonstrate that Cbl-mediated EGFR ubiquitinylation is required for efficient sorting of activated EGFR into the lysosome for its degradation. In contrast, neither Cbl nor Cbl-mediated ubiquitinylation are required for initial EGFR endocytosis.

Material and Methods

Reagents:

Biotin-EGF complexed with Alexa-Fluor-488-labeled streptavidin, and Alexa-Fluor-488-conjugated transferrin were obtained from Molecular Probes Inc. (www.molecularprobes.com). Opti-MEM I Reduced Serum Medium was from Invitrogen Corporation (www.invitrogen.com). The Fugene-6 reagent was obtained from Roche Molecular Biochemicals (www.roche.com).

Antibodies:

The antibodies used in this study were: mouse anti-EGFR monoclonal (mAb) 528 (IgG2a) from ATCC; rat anti-LAMP-1 mAb and rabbit polyclonal (pAb) anti-EGFR antibody (sc-03) from Santa Cruz Biotechnology, Inc. (www.scbt.com); anti-ubiquitin mAb P4G7 (IgG1) from Covance Research Products Inc. (www.covance.com); anti-phosphotyrosine mAb 4G10 (IgG2a) from Dr. Brian Druker (Oregon Health Sciences University, Portland, OR); and pAb anti-LAMP-1 (931B) (29) from Dr. Minoru Fukuda (The Burnham Institute, CA). Cy3-conjugated goat-anti-mouse IgG and Cy2-conjugated goat-anti-rabbit IgG secondary reagents were from Jackson Immuno-Research Laboratories Inc. (www.jacksonimmuno.com).

DNA constructs and mutants:

pAlterMAX-HA-Cbl and pAlterMAX-HA-Cbl-C3AHN (RING finger mutant) (30), and pJZenNeo-HA-Cbl retroviral expression construct (31) have been described

previously. The human EGFR cDNA insert from pAlterMAX-EGFR construct (17) was cloned into pcDNA3 to generate pcDNA3-EGFR, and into pMSCV-puro retroviral vector (www.clonetech.com) to generate pMSCV-puro-EGFR.

Cell lines:

The Chinese Hamster Ovary (CHO) cell line with a temperature-sensitive E1 ubiquitin-activating enzyme, CHO-Ts20 and its wildtype control cell line, CHO-E36 (32) (from Dr. Ger Strous, University Medical Center, Utrecht, The Netherlands) were grown in MEM-a medium supplemented with 10% fetal bovine serum, and penicillin/streptomycin (www.invitrogen.com). Mouse embryonic fibroblast (MEF) cells were derived using standard isolation methods (33) from day 13.5 $Cbl^{-/-}$ and littermate $Cbl^{+/+}$ embryos, from two separate $Cbl^{-/-}$ mouse backgrounds (34,35), followed by the 3T3 protocol from passage 3 to 25 (36). These cells were grown in MEM-a medium with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 0.1 mM non-essential amino acids, and penicillin/streptomycin. The MEF cells used here are independent of another pair that we have reported previously (37,38).

Transfections:

Transient or stable transfectants of CHO-Ts20, CHO-E36 expressing human EGFR (or its mutants) were generated by Fugene-6-mediated transfection of pcDNA3-EGFR, according to the manufacturer's instructions. Transfectants were selected in media containing 0.5 mg/ml G418 (www.invitrogen.com) and clones were analyzed for EGFR expression by immunoprecipitation and immunoblotting (described below).

Stable transfectants of $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs expressing human EGFR were established by retroviral infection as described previously (39). Cells were selected in puromycin (www.sigma-aldrich.com; 2.5 μg/ml and bulk transfectants were analyzed for EGFR expression using fluorescence-activated cell sorter (FACS) analysis with mAb 528 and immunoblotting with sc-03 anti-EGFR Ab.

Transfections were carried out with the Fugene-6 reagent. The amounts of input DNA are indicated in the figure legends. Cells were harvested at 48h after the addition of DNA precipitates.

EGF stimulation and preparation of cell lysates:

For EGF stimulation, cells were placed in starvation medium (growth medium containing 0.5% FBS) for 4-6h and then incubated with purified murine EGF (Catalog #E-4127, www.sigma-aldrich.com) for various lengths of time and at concentrations indicated in figure legends. Cells were rinsed with ice-cold PBS and lysed in 50 mM Tris (pH 7.5), 150 mM sodium chloride, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.07 trypsin inhibitor units/ml of aprotinin, and 1 μg/ml each of leupeptin, pepstatin, antipain, and chymostatin) (www.sigma-aldrich.com) (40). Lysate protein concentration was determined using the Bradford method (www.bio-rad.com).

Immunoprecipitation and immunoblotting:

These procedures have been described previously (14).

Confocal immunofluorescence microscopy:

Cells seeded on glass coverslips were stimulated with EGF as described above. Cell fixation and immunostaining was described previously (17). Cells were stained with the appropriate primary antibody (4 μg/ml anti-EGFR mAb 528 or 1:500 anti-LAMP-1 pAb in blocking buffer) and followed with 1:300 dilution of goat anti-mouse IgG (H+L) F(ab')2-Cy3 and anti-rabbit IgG F(ab')2-Cy2. Coverslips were mounted on glass slides using Fluoromount-G (www.emsdiasum.com). Confocal microscopy was carried out using a Leica TCS-NT Confocal Laser Scanning microscope fitted with krypton and argon lasers, as previously described (41).

Assessment of EGFR internalization:

Cells were grown on 10-cm tissue culture dishes to 70-80% confluence. Following serum starvation for 4-6h, the cells were incubated with 25 ng/ml Alexa-Fluor-488-conjugated EGF at 4°C for 30min, washed 3 times with cold PBS, and incubated at normal cell growth temperature for the indicated time points to allow internalization. The cells were placed on ice to stop internalization, rinsed 3 times with cold PBS and subjected to an acid wash (0.2 M acetic acid and 0.5 M NaCl, pH 2.8) for 5min. Non-internalized EGF was removed by 3 washes with PBS, and the cells were detached from tissue culture dishes using a rubber scraper. Cells were washed and suspended in FACS buffer (2% FBS and 0.01% sodium azide in PBS), and fixed by adding an equal volume of 4% formaldehyde/PBS. Fluorescence emission due to internalized EGF was detected by flow cytometry. Mean fluorescence intensity of cells after EGF binding but without the acid wash was set to 100%, percentage internalization was calculated after subtracting background (fluorescence of cells subjected to acid wash without allowing

internalization). Flow cytometry, data collection and analysis were performed on a FACSort machine using CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Each experiment was done in triplicate 3 times. Values are expressed as a percentage of initial EGF binding. The data was analyzed statistically with Prism (Graphpad Software, Inc.) by using student t-test and one-way ANOVA. All of the values from a representative experiment are plotted. The median is connected by a line, and the range from maximum to minimum is expressed as an error bar.

Downregulation of cell-surface EGFR:

Downregulation of EGFR from the cell surface was assessed as previously described (17).

Results and Discussion

Ligand-induced EGFR ubiquitinylation and degradation are impaired in cells lacking endogenous Cbl expression: Previous studies supporting a role for Cbl in EGFR ubiquitinylation and downregulation have exclusively used overexpression of wildtype Cbl or its dominant negative mutants (17,18,42). However, the role of endogenous Cbl in EGFR ubiquitinylation and degradation has not been addressed directly. To address this question, we derived two distinct pairs of $Cbl^{+/+}$ and $Cbl^{-/-}$ mouse embryo fibroblast cell lines (MEFs), using $Cbl^{-/-}$ mouse lines developed independently in the Bowtell and Gu laboratories (34,35); these cell lines are designated $Cbl^{+/+}$ (DB) and $Cbl^{-/-}$ (DB), and $Cbl^{+/+}$ (HG) and $Cbl^{-/-}$ (HG), respectively.

Western blotting of whole cell lysates confirmed the Cbl protein expression in the $Cbl^{+/+}$ MEFs but not in either of the $Cbl^{-/-}$ MEFs (Fig. 1A). The $Cbl^{-/-}$(DB) MEFs express low levels of a truncated Cbl protein (data not shown) representing a nonfunctional splice product, as previously reported (34,37). Since other Cbl family members, such as Cbl-b and Cbl-c, may also play a role in EGFR downregulation (43,44), we wished to determine the level of expression of Cbl-b and Cbl-c in these MEFs. To assess the expression of Cbl-b, we used an antibody (H454) that recognizes both Cbl and Cbl-b (determined using HA-tagged Cbl and Cbl-b proteins; data not shown). We found that Cbl-b protein was detectable in the lysates of $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs from which all Cbl protein had been immunodepleted using a Cbl-specific antibody (Fig. 1B, lanes 5-8); notably, the level of Cbl-b protein was substantially lower in the $Cbl^{-/-}$ (HG) MEFs. We also assayed for the presence of Cbl-c by Northern blot, as no antibody is currently

available; the Cbl-c mRNA levels were nearly undetectable in all MEFs and there was no compensatory increase in $Cbl^{-/-}$ cells (data not shown). While the reasons for reduced Cbl-b expression in the $Cbl^{-/-}$ (HG) MEFs are unknown, this trait was advantageous to assess the contribution of endogenous Cbl to EGFR downregulation. To analyze the impact of Cbl deficiency on EGFR downregulation, we used retroviral infection to derive stable transfectants of the $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs expressing human EGFR on the cell surface (Fig. 1C and 1D).

As Cbl is an EGFR-directed ubiquitin ligase (10-12), we first assessed the ligand-induced EGFR ubiquitinylation in EGFR-expressing $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs. Serum-starved cells were stimulated with EGF for various times, and anti-EGFR immunoprecipitates (IPs) were subjected to immunoblotting (IB) with an anti-ubiquitin antibody. As anticipated, ligand-dependent ubiquitinylation of EGFR was readily detected in both $Cbl^{+/+}$ MEF lines (DB and HG; Fig. 2A). Notably, ligand-induced EGFR ubiquitinylation in both $Cbl^{-/-}$ MEF lines was greatly reduced, indicating that endogenous Cbl is the major ubiquitin ligase involved in EGFR ubiquitinylation in MEFs. Importantly, anti-EGFR blotting of anti-EGFR IPs (Fig. 2A, second panel) or whole cell lysates (Fig. 2B) revealed that the ligand-induced loss of EGFR protein was substantially slower in both $Cbl^{-/-}$ MEFs; concomitantly, we observed a slower and less pronounced loss of phosphotyrosine (pY) signals on EGFR (Fig. 2A, bottom panel). The pattern of EGFR phosphorylation also indicates that impaired EGFR ubiquitinylation in $Cbl^{-/-}$ cells is not due to defective EGFR activation.

To establish that defective EGFR ubiquitinylation and degradation in $Cbl^{-/-}$ MEFs was due to the lack of Cbl expression, and not an artifact of cell line derivation, we

reconstituted Cbl expression in the EGFR-expressing $Cbl^{-/-}$ (HG) MEFs. IB of whole cell lysates demonstrated that retrovirus-mediated introduction of HA-tagged Cbl led to reconstitution of Cbl expression in $Cbl^{-/-}$ (HG) MEFs (Fig. 2C). Comparison of the HA-Cbl-transfected versus the vector-transfected cells demonstrated that reconstitution with Cbl fully restored the EGF-induced ubiquitinylation and degradation of EGFR (Fig. 2D and 2E). Thus, the defect in EGFR ubiquitinylation and degradation in $Cbl^{-/-}$ (HG) MEFs is solely due to lack of Cbl expression. Overall, the results with $Cbl^{-/-}$ MEFs and their Cbl-reconstituted derivatives establish a clear role for endogenous Cbl in ligand-induced EGFR ubiquitinylation and degradation.

EGFR internalization is unaltered in $Cbl^{-/-}$ MEFs: The precise site(s) of Cbl action in the endocytic trafficking of EGFR has not been clarified. Based on the formation of a Cbl-CIN85-endophilin complex, a recent set of studies concluded that Cbl plays an important role in the initial internalization of EGFR (25,45). In addition, an EGFR mutant unable to bind Grb2 was impaired in its ability to form an EGFR-Grb2-Cbl complex, and to undergo ligand-induced ubiquitinylation, and internalization, further suggesting a role for Cbl-mediated ubiquitinylation in EGFR internalization (26). However, whether the effects of the CIN85 mutant are due to the loss of a Cbl-CIN85 interaction or of other protein-protein interactions, or whether Cbl-EGFR interaction through Grb2 is required for EGFR internalization, have not been established. In fact, the N-terminal half of Cbl, which included only the TKB and RING finger domains and lacked both the CIN85 and Grb2 interaction sites, was sufficient to enhance the ubiquitinylation and degradation of EGFR (17,46,47). Moreover, overexpression of Cbl

did not enhance the EGFR internalization (42), and dominant-negative Cbl mutants blocked ubiquitinylation and degradation but not the internalization of EGFR (17,18). These discrepancies suggest that the impairment of EGFR internalization by the dominant interfering forms of CIN85 may not be through an interruption of the Cbl-CIN85 interaction but through another mechanism. Similarly, Cbl-Grb2 interaction may not be the basis for Grb2-regulated EGFR internalization, as Grb2 can also recruit a number of other proteins, such as RN-tre, to activated EGFR (48). To clarify the role of Cbl in EGFR internalization, we used the MEF system characterized above to directly establish if the loss of Cbl function had an obvious impact on ligand-induced internalization of EGFR. For this purpose, we assayed the acid-stable uptake of Alexa-Fluor-488-conjugated-EGF by MEFs.

To minimize the contribution of recycling and/or lysosomal degradation of internalized EGFR, we quantified the Alexa-488-EGF uptake in MEFs for relatively brief time periods (up to 10 min). We also used EGF at a relatively low concentration (25 ng/ml) that only saturates 40-50% of cell surface EGFR in our system (data not shown). The rate of initial EGFR internalization in $Cbl^{-/-}$ (HG) MEFs was comparable to that in $Cbl^{+/+}$ (HG) MEFs (Fig. 3A, $P>0.05$). Furthermore, the rate of initial internalization of EGFR in Cbl-reconstituted and vector-transfected $Cbl^{-/-}$ (HG) MEFs was essentially identical (Fig. 3C, left panel, $P>0.05$), even though the downregulation of EGFR at later time points, which reflects a net balance of internalization, recycling and lysosomal degradation, was dramatically slower in $Cbl^{-/-}$ (HG) MEFs compared to Cbl-reconstituted MEFs (Fig. 3C, right panel, $P<0.001$). Unimpaired EGFR internalization in $Cbl^{-/-}$ (HG) MEFs was further confirmed by the immunofluorescence staining of EGFR after EGF

stimulation. The pattern of EGFR-staining endocytic vesicles 10 min after EGF stimulation was comparable in $Cbl^{-/-}$ and $Cbl^{+/+}$ (HG) MEFs (Fig. 3B).

The combination of unimpaired initial internalization and reduced EGFR degradation (and downregulation) in $Cbl^{-/-}$ MEFs indicates that Cbl function is critical at a post-internalization step in EGFR downregulation but not at the internalization step.

CHO-Ts20 cells demonstrate that the ubiquitin pathway is essential for EGFR degradation but dispensable for internalization: Previous analyses using overexpression of wildtype or mutant forms of Cbl have demonstrated a tight correlation between the Cbl-regulated ubiquitinylation and degradation of EGFR (49). This correlation is further strengthened by studies of EGFR mutants unable to bind to Cbl (50), and analyses of Cbl-deficient MEFs presented above. However, the questions of whether Cbl-mediated EGFR degradation proceeds through ubiquitinylation and whether the ubiquitin pathway is required to target EGFR for lysosomal degradation have not been directly addressed. To clarify these issues, we utilized a CHO cell line with a temperature-sensitive ubiquitin-activating (E1) enzyme, which results in a conditional defect in protein ubiquitinylation (51).

As CHO cells lack EGFR, the CHO cell line with mutant E1 (CHO-Ts20) and its wildtype counterpart (CHO-E36) were stably transfected with human EGFR. Transient overexpression of wildtype Cbl, in comparison with vector control, enhanced the ubiquitinylation of EGFR in Ts20-EGFR cells (Fig. 4A, compare lanes 2 and 4). In contrast, the overexpression of a Cbl RING finger domain mutant, Cbl-C3AHN, suppressed the ligand-induced ubiquitinylation (Fig. 4A, lane 6) and degradation of

EGFR (Fig. 4B, lanes 7-9). Thus, both ubiquitinylation and degradation of EGFR in CHO-Ts20 cells are dependent on endogenous Cbl.

To directly assess the impact of inhibiting ubiquitinylation in CHO-Ts20 cells on EGFR degradation, we compared Ts20-EGFR and E36-EGFR (control) cell lines at 30°C, the permissive temperature for ubiquitinylation, versus 42°C, the non-permissive temperature. When assayed at 30°C, the ligand-induced EGFR ubiquitinylation was observed in both Ts20-EGFR and E36-EGFR cells (Fig. 5A and 5B, lanes 1-3 and 7-9). In contrast, ligand-induced EGFR ubiquitinylation was markedly attenuated in Ts20-EGFR cells shifted to 42°C (Fig. 5A, lanes 10-12); as a control, the ubiquitinylation of EGFR in E36-EGFR cells was unaffected by the temperature shift (Fig. 5A, lanes 4-6). Analysis of EGFR protein levels over a longer time course of EGF stimulation demonstrated that the kinetics of EGFR degradation in E36-EGFR cells was similar at 30°C versus 42°C (Fig. 5B, compare lanes 1-4 and 5-8). In contrast, the degradation of EGFR in Ts20-EGFR cells was retarded when these cells were shifted to 42°C to block ubiquitinylation (Fig. 5B, compare lanes 9-12 and 13-16). The requirement of Cbl for EGFR degradation, together with the inhibition of EGFR degradation upon blockade of the ubiquitinylation pathway in Ts20-EGFR cells, supports the view that it is the ubiquitin ligase activity of Cbl rather than another function that is essential in Cbl-mediated EGFR degradation. Interestingly, in both $Cbl^{-/-}$ MEF cell lines that we utilized, we observed a low level of residual EGFR ubiquitinylation upon ligand stimulation. Whether this is due to a low level of Cbl-b expression or due to an unrelated ubiquitin ligase capable of targeting EGFR for ligand-induced ubiquitinylation, remains to be investigated. The residual ubiquitinylation could account for the EGFR degradation that

is still observed. Alternatively, the continued EGFR degradation could reflect the contribution of other mechanisms, such as those mediated by the dileucine motifs, to endosomal sorting of EGFR (52).

Our finding that the initial internalization of EGFR was intact in $Cbl^{-/-}$ MEFs suggested that Cbl and Cbl-mediated EGFR ubiquitinylation was dispensable for EGFR internalization, but left open the possibility that ubiquitinylation of another protein(s) may mediate the internalization. In this regard, EGF-inducible ubiquitinylation of endocytic proteins, such as Eps15 (53) and CIN85 (54), has been previously demonstrated, although the role of the ubiquitinylation in EGFR internalization remains to be established. Ubiquitin has been clearly recognized as a receptor internalization motif in yeast (20,21), while studies of the growth hormone receptor in mammalian cells indicate that ubiquitinylation of an unknown non-receptor component may be crucial for internalization (22). The Ts20-EGFR system provided a suitable system to test if the ubiquitin pathway was also essential for initial internalization of EGFR. Therefore, we also examined the EGFR internalization in Ts20-EGFR and E36-EGFR cells, using the assay described above. Similar to control E36-EGFR cells (Fig. 6, left panel, $P>0.05$), the rate of initial EGFR internalization in Ts20-EGFR cells grown at 30°C and 42°C was comparable (Fig. 6B, right panel, $P>0.05$). Thus, the ubiquitinylation machinery is dispensable for the initial internalization step in the endocytic traffic of EGFR.

Our results indicate that neither EGFR ubiquitinylation nor the monoubiquitinylation of other proteins, such as Eps15 and CIN85, is required for EGFR internalization. Dikic and colleagues have, however, observed that a dominant negative mutant of CIN85, which is not monoubiquitinylated by Cbl upon EGF stimulation,

impairs the internalization and degradation of EGFR (23, 45). It is likely that the dominant negative form of CIN85 fails to interact with other partners, which may relate to its role at the internalization step. Furthermore, Dikic and colleagues observed that the Cbl-dependent monoubiquitinylation of CIN85 primarily occurred after EGFR endocytosis (23), which suggests a role for ubiquitinylated CIN85 at a post-endocytic step, consistent with our conclusions.

Our results do not exclude the possibility that ubiquitinylation can function as an endocytic signal. In fact, monoubiquitinylation of EGFR is an enough signal for EGFR internalization (55). Our results do indicate, though, that this cannot be the sole mechanism for initial internalization, and that other mechanisms can fully support the internalization process in the absence of the ubiquitinylation of EGFR and any accessory proteins. The molecular nature of these additional signals remains to be fully elucidated. Several endocytic motifs have been identified in EGFR, including the dileucine-based motif (56) and the tyrosine-based AP-2 binding motif (57). However, mutational analyses suggest that none of these motifs is essential for internalization (56,57). It is likely that a complex RTK such as EGFR has evolved multiple redundant mechanisms to ensure internalization, as this is a key regulatory process.

The internally controlled Ts20-EGFR and E36-EGFR cell pair also provided an opportunity to assess the nature of the endocytic compartment(s) where Cbl functions and where ubiquitinylation plays a decisive role in the endocytic trafficking of internalized EGFR. For this purpose, we assessed the colocalization of the internalized EGFR with selected endocytic markers, using confocal microscopy. In both E36-EGFR and Ts20-EGFR cells grown at 30^{o}C, the EGFR-containing vesicles were peripherally distributed

at 5 min after EGF stimulation (Fig. 7A, B1 and E1) and gradually moved near the center of the cells, forming larger clusters by 30 min (Fig. 7A, C1 and F1). When E36-EGFR and Ts20-EGFR cells were compared at 42°C, the EGFR staining pattern at 5 min of EGF stimulation was similar (Fig. 7B, compare B1 and E1), consistent with the unaltered EGFR internalization seen in the internalization assay. Notably, however, the distribution of EGFR containing vesicles in Ts20-EGFR versus E36-EGFR cells grown at 42°C and stimulated for 30 min was quite distinct; these vesicles failed to move near the center and remained near the periphery in Ts20-EGFR cells (Fig. 7B, compare C1 and F1).

To characterize the EGFR-containing endosomal compartments, we either loaded the cells with fluorescent transferrin to mark the early/recycling endosomes, or carried out double staining for EGFR (Red) and LAMP-1 (36), a late endosome/lysosome marker; the cells were then analyzed using confocal microscopy. At 5 min of EGF stimulation at 30°C, the internalized EGFR in peripherally distributed vesicles mostly colocalized with transferrin (Fig. 7A, B3 and B4; E3 and E4; yellow); this colocalization was lost by 30 min of stimulation, when the EGFR was predominantly centrally clustered (Fig. 7A, C3 and C4; F3 and F4; red). However, EGFR in these vesicles colocalized with LAMP-1 (Fig. 8, B3 and B4; E3 and E4; yellow), indicating that a proportion of internalized EGFR underwent a time-dependent endocytic transport from early to late endosomes. This pattern remained unaltered when the E36-EGFR control cells were examined at 42°C (Fig. 7B, C3 and C4). In contrast, when Ts20-EGFR cells were grown at 42°C to disrupt ubiquitinylation, the internalized EGFR remained colocalized with transferrin at 30 min of EGF stimulation (Fig. 7B, F3 and F4; yellow), and showed a

lower degree of colocalization with LAMP-1 (Fig. 8, F3 and F4). These results indicate that ubiquitinylation is crucial for an early endosome to late endosome sorting step in the endocytic trafficking of ligand-stimulated EGFR.

The simplest interpretation of these results is that Cbl-mediated ubiquitinylation of EGFR (and/or other accessory proteins) serves as an endosomal sorting signal for EGFR delivery from early to late endosome/lysosome, and that ubiquitin modification serves as an essential signal at this step to ensure the efficient delivery of EGFR into the degradative compartments of the endocytic machinery. This model is consistent with the previous finding that ubiquitinylation and proteasome activity is needed for EGFR transfer into internal vesicle of MVBs (28). It is also compatible with the genetically defined role of ubiquitinylation of yeast transmembrane receptors for sorting into the inner vesicles of the MVB (58,59). In fact, yeast studies have identified endosomal sorting of receptor traffic (ESCRTs) complexes, such as ESCRT-1 (60), that function as ubiquitin recognizing proteins within the endocytic system. Deletion of TSG-101, one of the ESCRT-1 complex components in mammals, retards EGFR degradation and causes accumulation of ubiquitinylated proteins on endosomes (61). Furthermore, the hepatocyte growth factor receptor substrate (Hrs), the mammalian counterpart of the yeast ESCRT-2 protein also has a ubiquitin interacting motif (UIM) (53,62,63). A truncated Hrs protein lacking the UIM impaired EGFR degradation in *Drosophila*, by affecting endosome membrane invagination and MVB formation (64). Thus, Cbl-mediated ubiquitinylation could represent the receptor modification required for recognition by mammalian ESCRT proteins to facilitate endosomal sorting of EGFR to lysosomes. Whether or not the ubiquitin modification provides the signal for delivering

EGFR into the inner vesicles of the MVB in mammalian cells, and the regulatory control of this process, is an important area of future investigation.

In conclusion, our studies utilizing two distinct and independent experimental systems provide strong evidence that Cbl-mediated ubiquitinylation is essential for efficient ligand-induced degradation of EGFR, but is dispensable for initial receptor internalization. Future studies should address whether this dichotomy is a general feature of Cbl regulation of RTKs and other transmembrane receptors.

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Abbreviations:

CHO, Chinese hamster ovary; EGFR, epidermal growth factor receptor; IP, immunoprecipitate; IB, immunoblot; mAb, monoclonal antibody; LAMP, lysosome-associated membrane protein; MEF, mouse embryonic fibroblast; PAb, rabbit polyclonal antibody; PBS, phosphate-buffered saline; RTK, receptor tyrosine kinases; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WT, wildtype.

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Figure legends:

Fig. 1. Characterization of $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs. A. Lack of Cbl expression in $Cbl^{-/-}$ MEFs. 50 μg aliquots of cell lysates of HG and DB MEFs were resolved by SDS-PAGE and subjected to IB with an anti-Cbl antibody. The faster migrating band in $Cbl^{-/-}$ (DB) cell line is a truncated nonfunctional protein. **B.** Expression of Cbl-b relative to Cbl in MEFs, analyzed with a Cbl/Cbl-b cross-reactive antibody H454. 1 mg aliquots of cell lysates were subjected to IP with an anti-Cbl-specific antibody (Santa Cruz C-15, lanes 1-4). Alternatively, the same amounts of lysates were first subjected to two rounds of anti-Cbl IP, and immunodepletion of Cbl was confirmed by IB (not shown). These immunodepleted lysates were subjected to IP with H454 antibody (lanes 5-8) to immunoprecipitate Cbl-b. Both sets of IPs were subjected to IB with H454 antibody (recognizes both Cbl and Cbl-b; data not shown). Note reduced Cbl-b signal in $Cbl^{-/-}$ (HG) MEFs (lane 8). **C and D.** Analysis of EGFR expression on $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs. $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs were stably transfected with EGFR, or vector (-) using retroviral infection. 50 μg aliquots of cell lysate proteins were immunoblotted with an anti-EGFR Ab (sc-03) (C). Alternatively, cells were trypsinized and stained with an anti-EGFR mAb (528, thin line) or an isotype control antibody (anti-Syk; thick line) followed by FACS analysis to quantify the cell surface expression of EGFR (D). The numbers in each box represent mean fluorescence intensity (arbitrary values) of anti-EGFR staining.

Fig. 2. Impairment of EGFR ubiquitinylation and degradation in $Cbl^{-/-}$ MEFs, and reversal of these defects by reconstitution of Cbl expression. A. Impaired EGFR

ubiquitinylation in $Cbl^{-/-}$ MEFs. The MEFs were stimulated with EGF (100 ng/ml) for the indicated times (min) prior to lysis. Anti-EGFR (mAb 528) IPs from 2mg aliquots of cell lysates were serially immunoblotted with anti-ubiquitin (upper panel), anti-EGFR (sc-03; middle panel) and anti-pY (4G10) (lower panel) antibodies. Ubiquitinylated EGFR is indicated. **B.** Delayed EGFR degradation in $Cbl^{-/-}$ MEFs. The cell lysates were prepared as in A, and 50 μg aliquots of cell lysates were immunoblotted with anti-EGFR antibody sc-03. The relative EGFR signals were quantified by densitometry using the Scion Image software (Scion Corp., Maryland), and are depicted as a proportion of signals observed with unstimulated cell lysates (set as 1). **C-E.** Reversal of defective EGFR ubiquitinylation and degradation in $Cbl^{-/-}$ MEFs by Cbl reconstitution. Retroviral infection was used to derive HA-Cbl and vector transfected $Cbl^{-/-}$ (HG) MEFs. 50 μg aliquots of lysates of these cells, as well as untransfected or vector-transfected $Cbl^{+/+}$ MEFs, were immunoblotted with an anti-Cbl antibody (C). The relative levels of Cbl expression, as determined by densitometry, are indicated at the bottom. The vector-transfected or Cbl-reconstituted $Cbl^{-/-}$ (HG) MEFs were stimulated with EGF for the indicated times prior to cell lysis. 2 mg aliquots of lysates were used for anti-EGFR IP followed by serial anti-ubiquitin (upper panel) and anti-EGFR immunoblotting (lower panel) (D). 50 μg aliquots of the same lysates were directly immunoblotted with anti-EGFR antibody (E) to assess EGFR degradation. Relative EGFR signals were determined by densitometry.

Fig. 3. Unimpaired internalization but reduced downregulation of EGFR in $Cbl^{-/-}$ MEFs. A. EGFR internalization in $Cbl^{+/+}$, $Cbl^{-/-}$ MEFs. The internalization assay is

described in Materials and Methods. **B.** Internalization of EGFR as determined by immunofluorescence staining as described in Materials and Methods. Cells were either left unstimulated or stimulated with EGF (25 ng/ml for 10min). Internalization is indicated by the accumulation of intracellular endocytic vesicles staining for EGFR (negative controls are not shown). **C.** EGFR internalization and downregulation in $Cbl^{-/-}$ and Cbl-reconstituted $Cbl^{-/-}$ MEFs. Internalization assay is described as in A. For downregulation, cells were either left unstimulated or stimulated with EGF as in B. The levels of EGFR on the cell surface were quantified by FACS analysis after immunostaining with anti-EGFR Ab 528 as described in Materials and Methods. EGFR levels remaining on the cell surface are represented as a percentage of EGFR levels (mean fluorescence intensity) without EGF stimulation. Every experiment was done in triplicate 3 times. All of the three values from one representative experiment are plotted. The median is connected by a line, and the range from maximum to minimum is expressed as an error bar.

Fig. 4. Ubiquitinylation and degradation of EGFR in CHO-Ts20 cells is mediated by Cbl. A. Wildtype Cbl enhances while the Cbl RING finger mutant inhibits EGFR ubiquitinylation. CHO-Ts20-EGFR cells were transiently transfected with the pAlterMAX vector or constructs encoding HA-Cbl or HA-Cbl-C3AHN (RING finger mutant). Cells were grown at $30^{o}C$ and either left unstimulated or stimulated for 10min prior to lysis. 1 mg aliquots of cell lysates were subjected to anti-EGFR IPs followed by serial anti-ubiquitin (top panel) and anti-EGFR (bottom panel) IB. **B.** Wildtype Cbl enhances while the Cbl-C3AHN inhibits EGFR degradation. Cell lysates at various times

following EGF stimulation were prepared as in A, and 50 μg aliquots of lysates were subjected to anti-EGFR IB. The relative EGFR signals (no EGF = 1) were determined by densitometry and are indicated at the bottom.

Fig. 5. Conditional impairment of EGFR ubiquitinylation and degradation in CHO-Ts20 cells. A. Lack of EGFR ubiquitinylation at 42°C in CHO-Ts20-EGFR cells. Control CHO-E36 cells (Left panel) and mutant E1-expressing CHO-Ts20 cells (Right panel) were stably transfected with human EGFR and clones expressing EGFR were identified by IB. The cells were plated at 30°C and then either continued at the same temperature (Lane 1-3 and 7-9) or shifted to 42°C (lane 4-6 and 10-12) for 4 hours, prior to stimulation with 100 ng/ml EGF for the indicated times and then lysed. 1 mg aliquots of cell lysates were subjected to anti-EGFR IPs and serial immunoblotting with anti-ubiquitin (top panel), anti-EGFR (middle panel) and anti-pY (bottom panel) Abs. **B.** Reduced EGFR degradation at 42°C in CHO-Ts20-EGFR cells. Cell lysates at various times following EGF stimulation were prepared as in A, and 50 μg aliquots of lysates were subjected to anti-EGFR IB. The relative EGFR signals (no EGF = 1) were determined by densitometry and are indicated at the bottom.

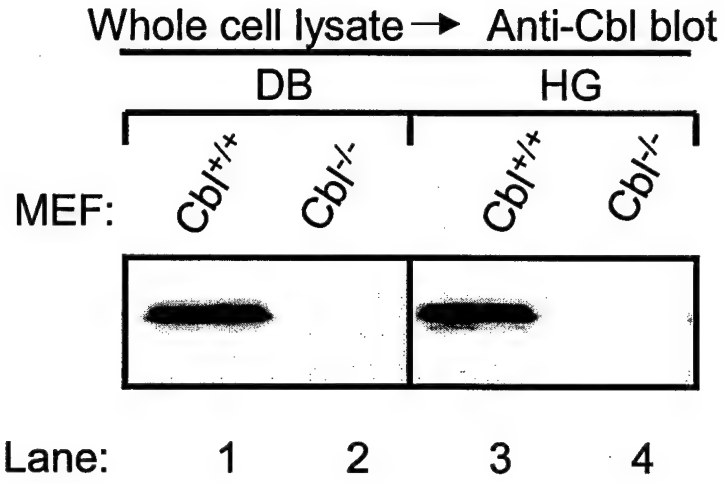
Fig. 6. Unimpaired EGFR internalization in CHO-Ts20 cells. Cells were grown at 30°C, and then either continued at the same temperature or shifted to 42°C, as indicated. Cells were allowed to bind Alexa-Fluor488-conjugated EGF at 4°C for 30min, and then returned to 37°C to allow EGF internalization. Every experiment was done in triplicate 3 times. The percentage of internalized EGF was calculated and plotted as in Fig. 3.

Fig. 7. Conditional alteration of internalized EGFR localization and its colocalization with transferrin receptor in CHO-Ts20 cells. Cells were either maintained at 30°C (A) or were shifted to 42°C (B), as in Fig. 6, and stimulated with EGF for the indicated times. Cells were loaded with Alexa-Fluor488-conjugated transferrin (36) for 5min prior to harvesting to visualize the early endosome/recycling endosome compartment. Cells were fixed, permeabilized and stained with anti-EGFR (mAb 528) followed by Cy3-conjugated anti-mouse antibody (red), and analyzed by confocal microscopy. Colocalization is indicated by yellow coloration in merged images.

Fig. 8. Conditional impairment of EGFR localization to late endosome/lysosome in CHO-Ts20 cells. Cells were processed as in Fig. 8 and stained with anti-EGFR antibody (visualized with Cy3-conjugated anti-mouse antibody; red) and rabbit anti-LAMP-1 antibody (late endosome/lysosome marker; visualized with Cy2-conjugated goat anti-rabbit antibody; green), and analyzed by confocal microscopy.

Fig. 1

A


Whole cell lysate → Anti-Cbl blot
DB
HG
MEF:
Cbl+/+
Cbl-/-
Cbl+/+
Cbl-/-
Lane:
1
2
3
4

B

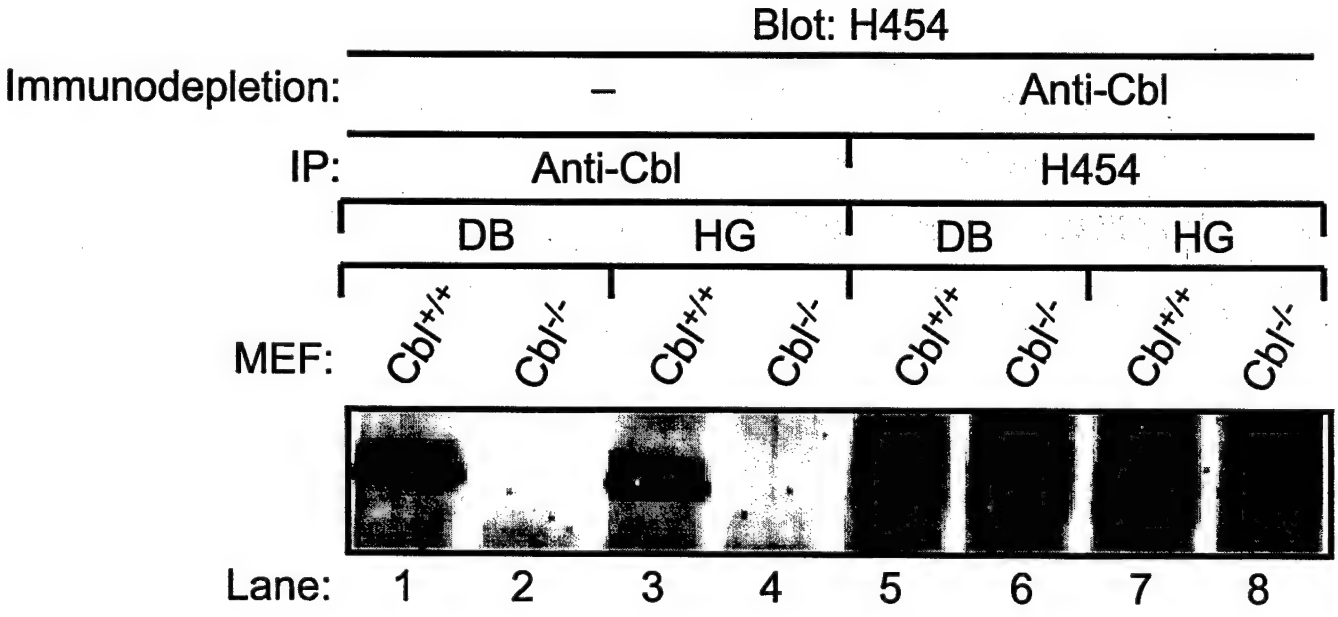
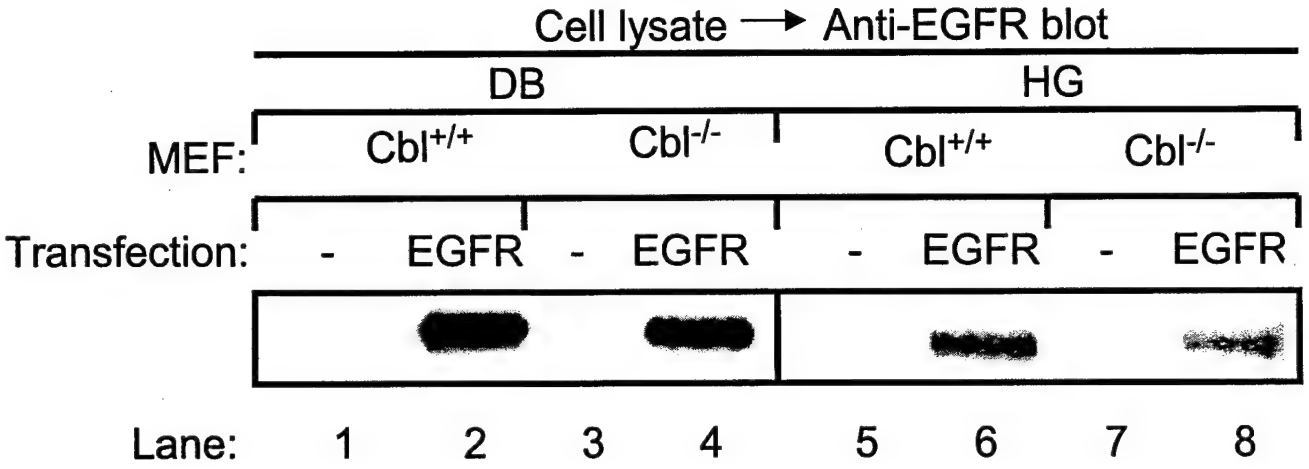

Blot: H454
Immunodepletion:
–
Anti-Cbl
IP:
Anti-Cbl
H454
DB
HG
DB
HG
MEF:
Cbl+/+
Cbl-/-
Cbl+/+
Cbl-/-
Cbl+/+
Cbl-/-
Cbl+/+
Cbl-/-
Lane:
1
2
3
4
5
6
7
8

Fig. 1-con't.

C


Cell lysate → Anti-EGFR blot
DB
HG
MEF:
Cbl+/+
Cbl-/-
Cbl+/+
Cbl-/-
Transfection:
-
EGFR
-
EGFR
-
EGFR
-
EGFR
Lane:
1
2
3
4
5
6
7
8

D

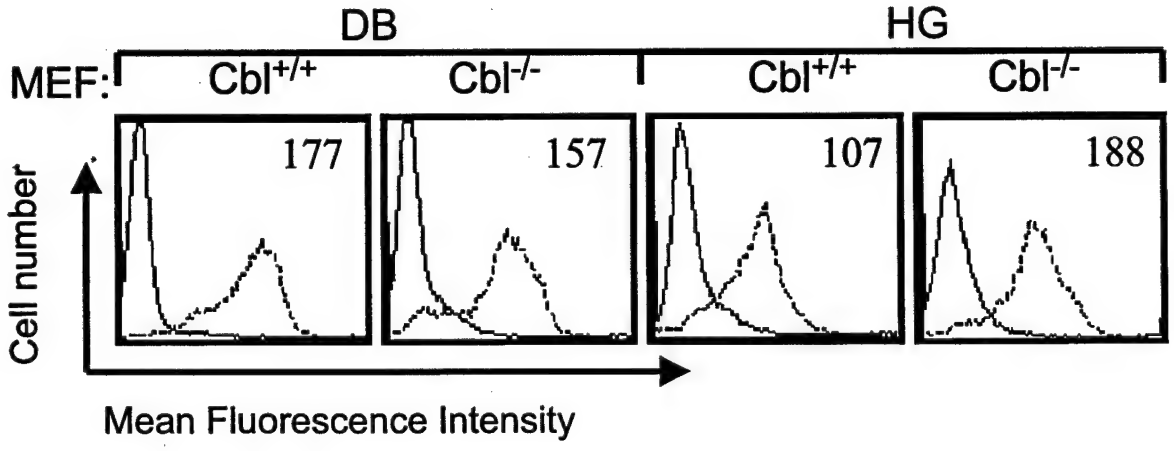
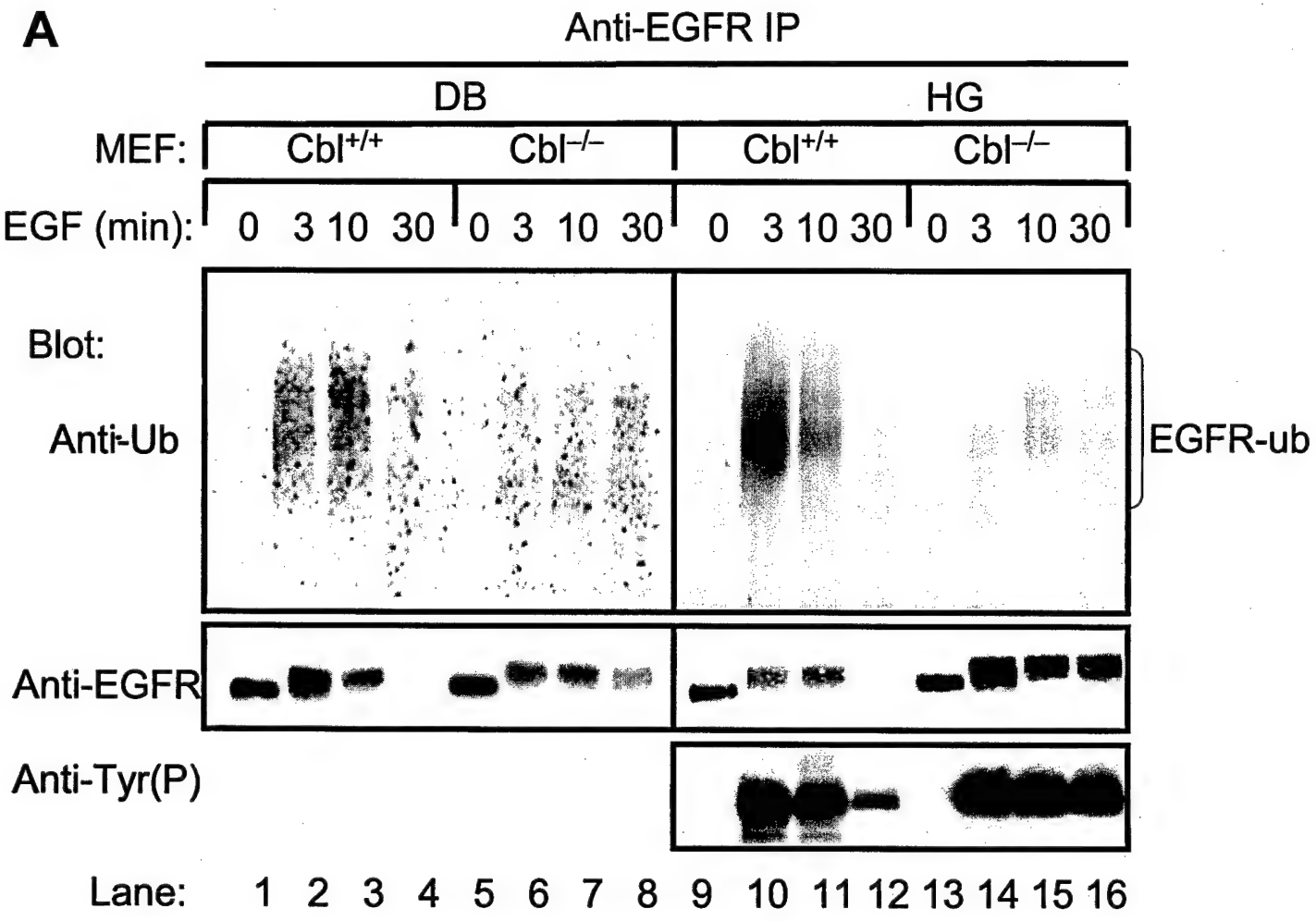

DB
HG
MEF:
Cbl+/+
Cbl-/-
Cbl+/+
Cbl-/-
177
157
107
188
Cell number
Mean Fluorescence Intensity

Fig. 2


A
Anti-EGFR IP
DB
HG
MEF:
Cbl+/+
Cbl−/−
Cbl+/+
Cbl−/−
EGF (min):
0 3 10 30 0 3 10 30 0 3 10 30 0 3 10 30
Blot:
Anti-Ub
EGFR-ub
Anti-EGFR
Anti-Tyr(P)
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

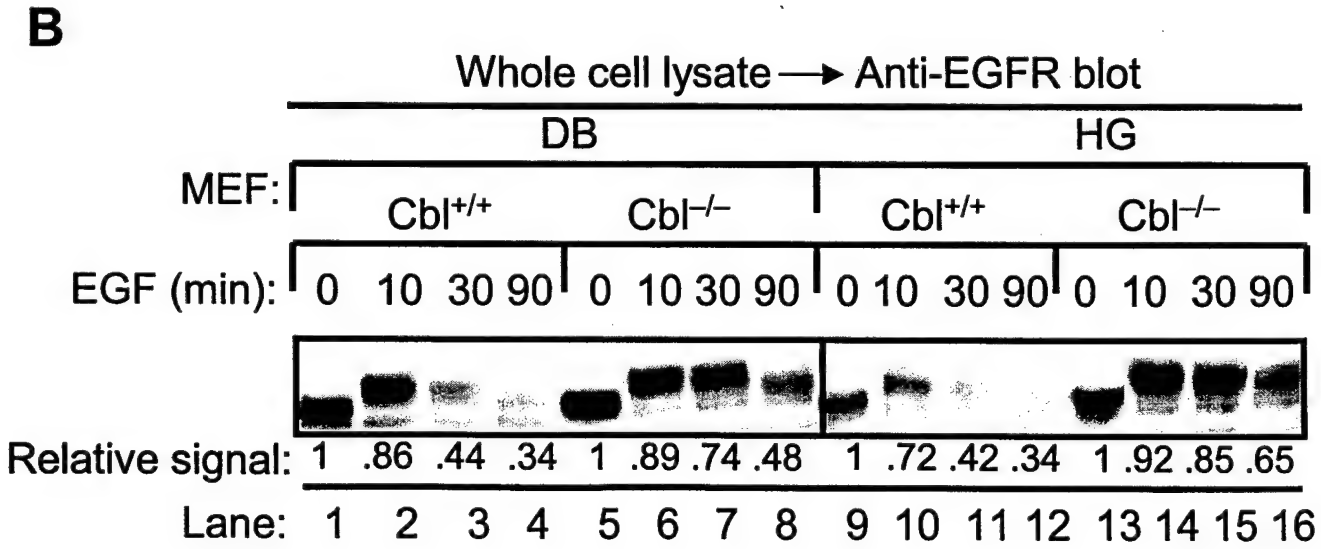

B
Whole cell lysate → Anti-EGFR blot
DB
HG
MEF:
Cbl+/+
Cbl−/−
Cbl+/+
Cbl−/−
EGF (min):
0 10 30 90 0 10 30 90 0 10 30 90 0 10 30 90
Relative signal:
1 .86 .44 .34 1 .89 .74 .48 1 .72 .42 .34 1 .92 .85 .65
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 2-con't.

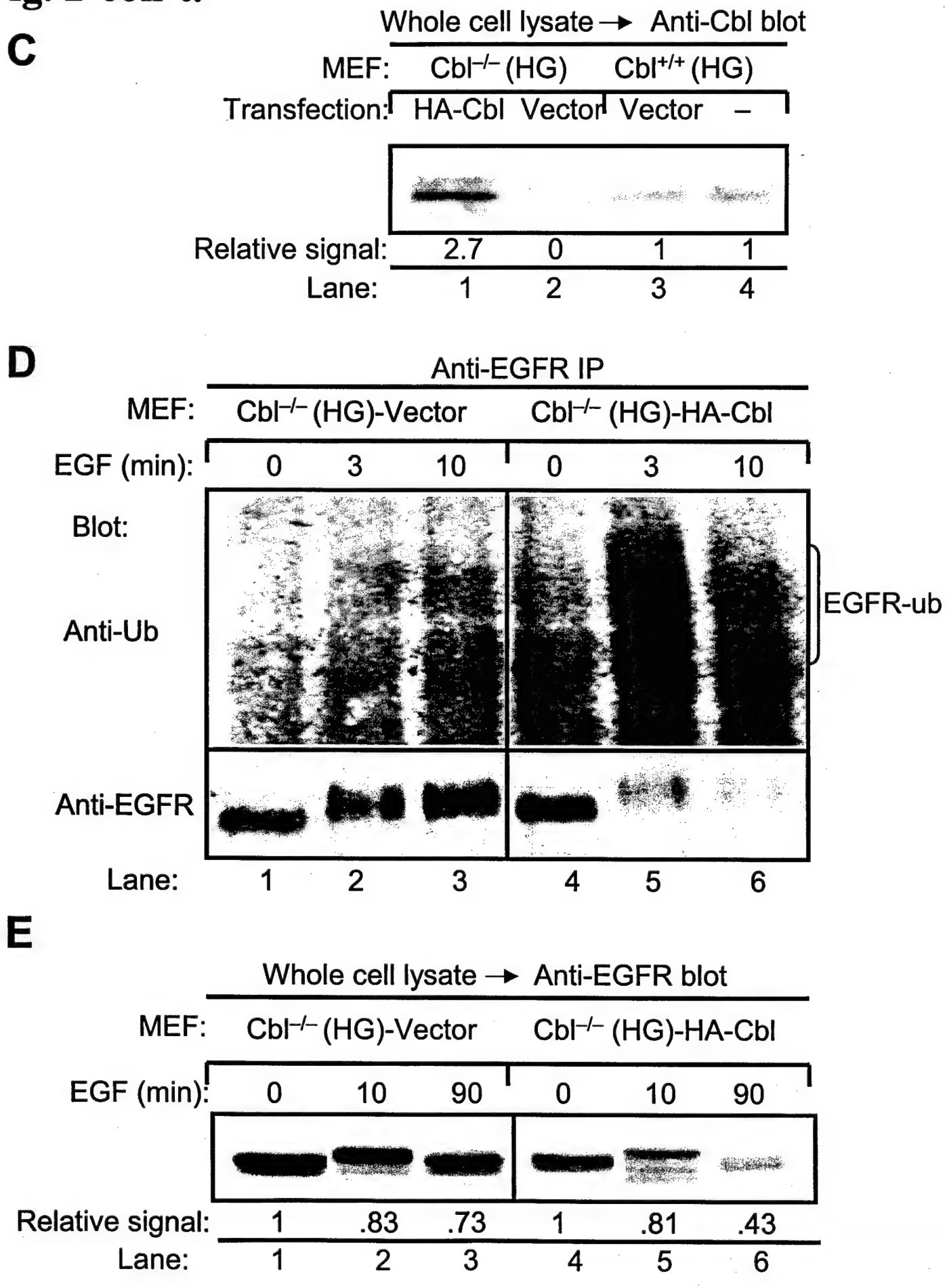
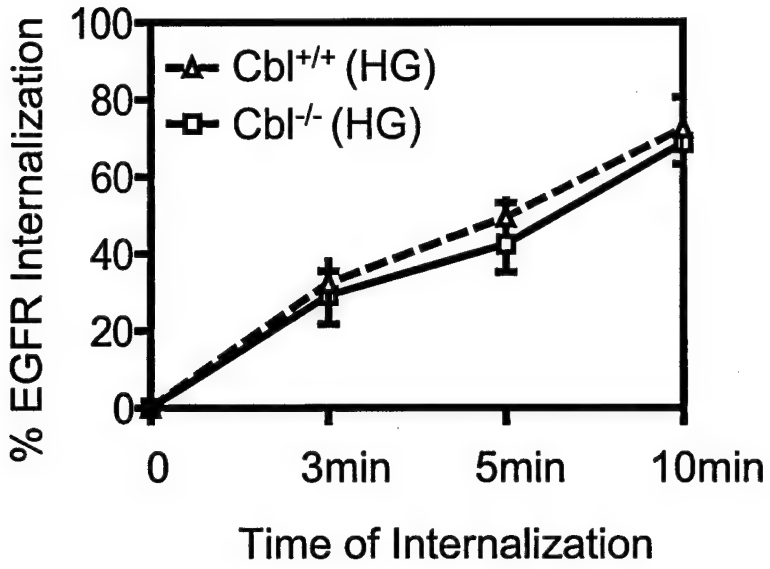

C
Whole cell lysate → Anti-Cbl blot
MEF: Cbl−/− (HG) Cbl+/+ (HG)
Transfection: HA-Cbl Vector Vector –
Relative signal: 2.7 0 1 1
Lane: 1 2 3 4
D
Anti-EGFR IP
MEF: Cbl−/− (HG)-Vector Cbl−/− (HG)-HA-Cbl
EGF (min): 0 3 10 0 3 10
Blot:
Anti-Ub
EGFR-ub
Anti-EGFR
Lane: 1 2 3 4 5 6
E
Whole cell lysate → Anti-EGFR blot
MEF: Cbl−/− (HG)-Vector Cbl−/− (HG)-HA-Cbl
EGF (min): 0 10 90 0 10 90
Relative signal: 1 .83 .73 1 .81 .43
Lane: 1 2 3 4 5 6

Fig. 3

A


100
80
60
40
20
0
% EGFR Internalization
Cbl+/+ (HG)
Cbl-/- (HG)
0
3min
5min
10min
Time of Internalization

B

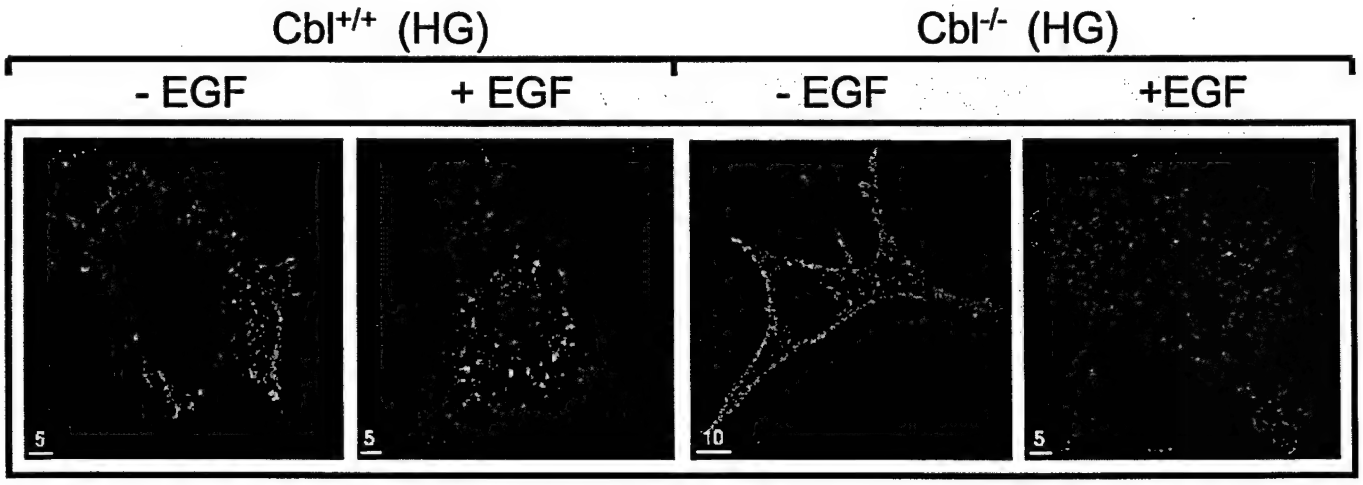

Cbl+/+ (HG)
Cbl-/- (HG)
- EGF
+ EGF
- EGF
+EGF
5
5
10
5

Fig. 3-con't.

C

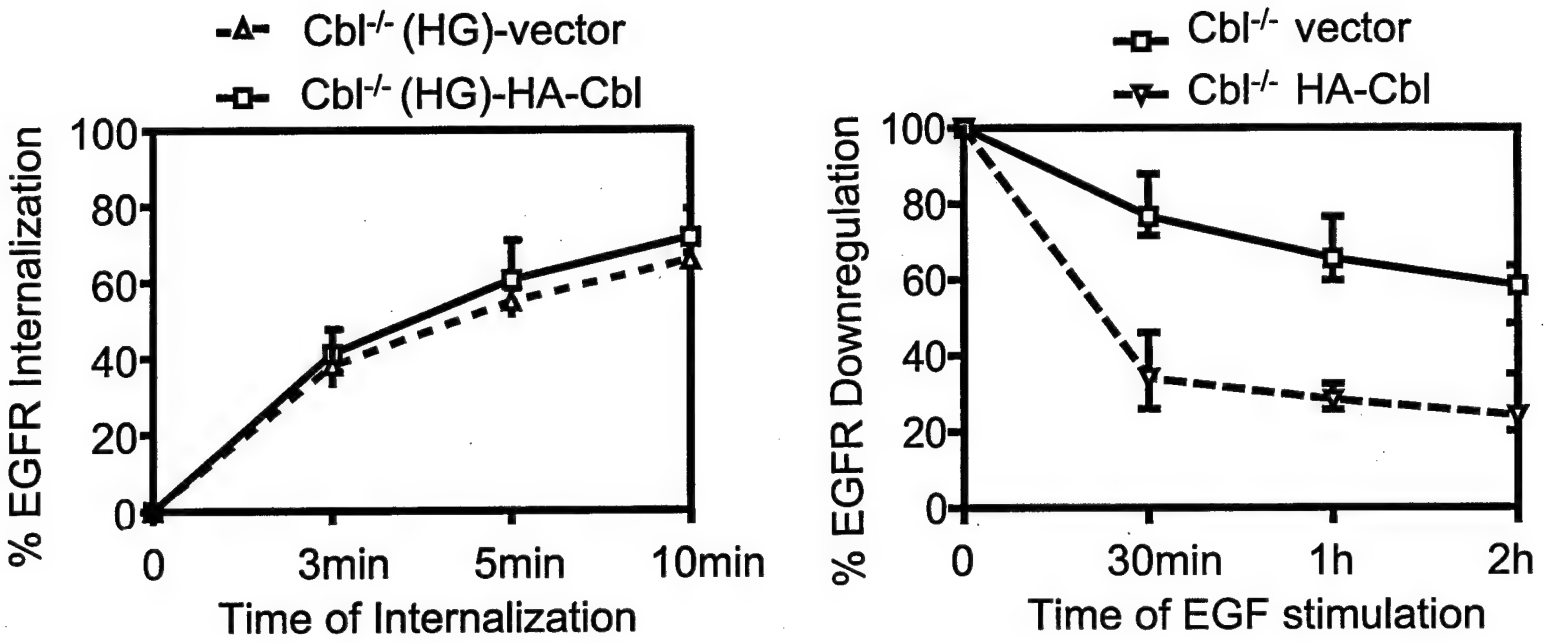
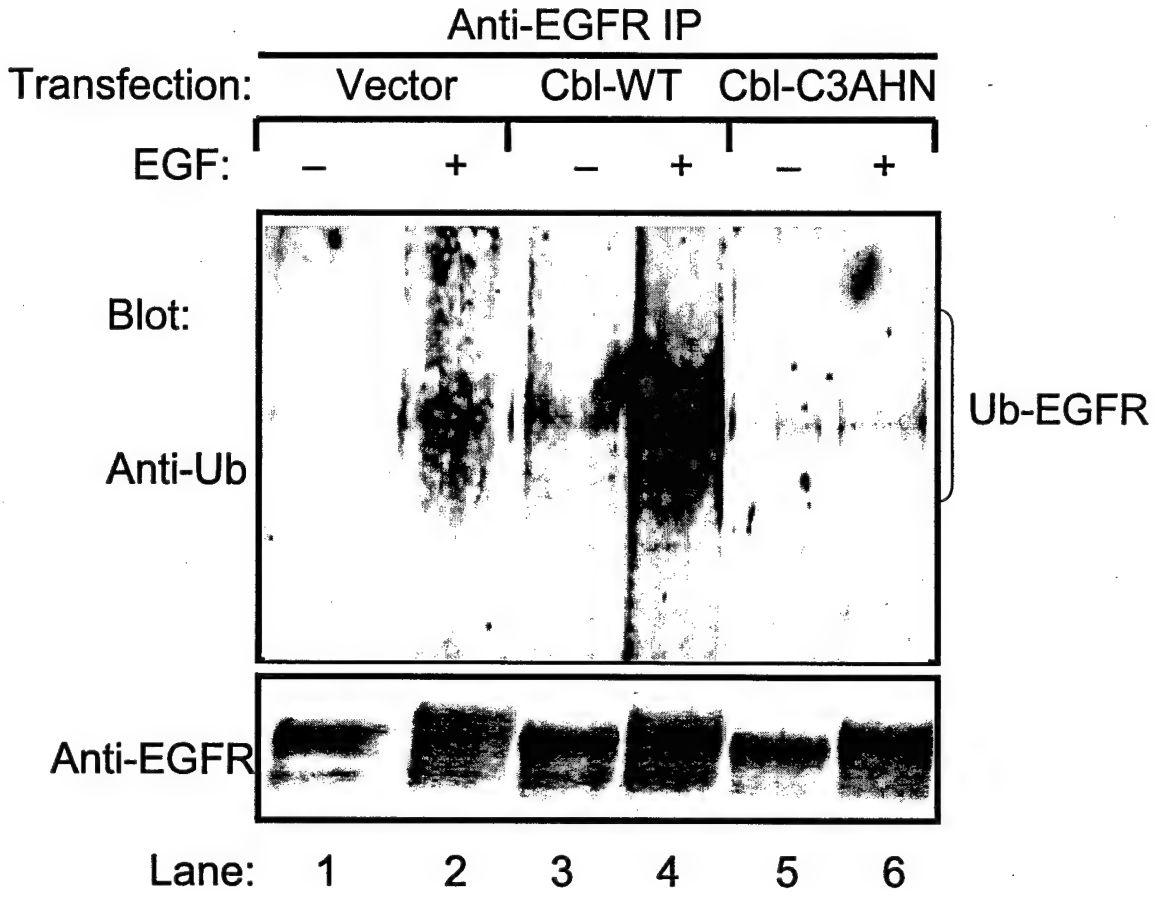

Cbl-/- (HG)-vector
Cbl-/- (HG)-HA-Cbl
% EGFR Internalization
100
80
60
40
20
0
0
3min
5min
10min
Time of Internalization
Cbl-/- vector
Cbl-/- HA-Cbl
% EGFR Downregulation
100
80
60
40
20
0
0
30min
1h
2h
Time of EGF stimulation

Fig. 4

A


Anti-EGFR IP
Transfection:
Vector
Cbl-WT
Cbl-C3AHN
EGF:
– + – + – +
Blot:
Anti-Ub
Ub-EGFR
Anti-EGFR
Lane:
1 2 3 4 5 6

B

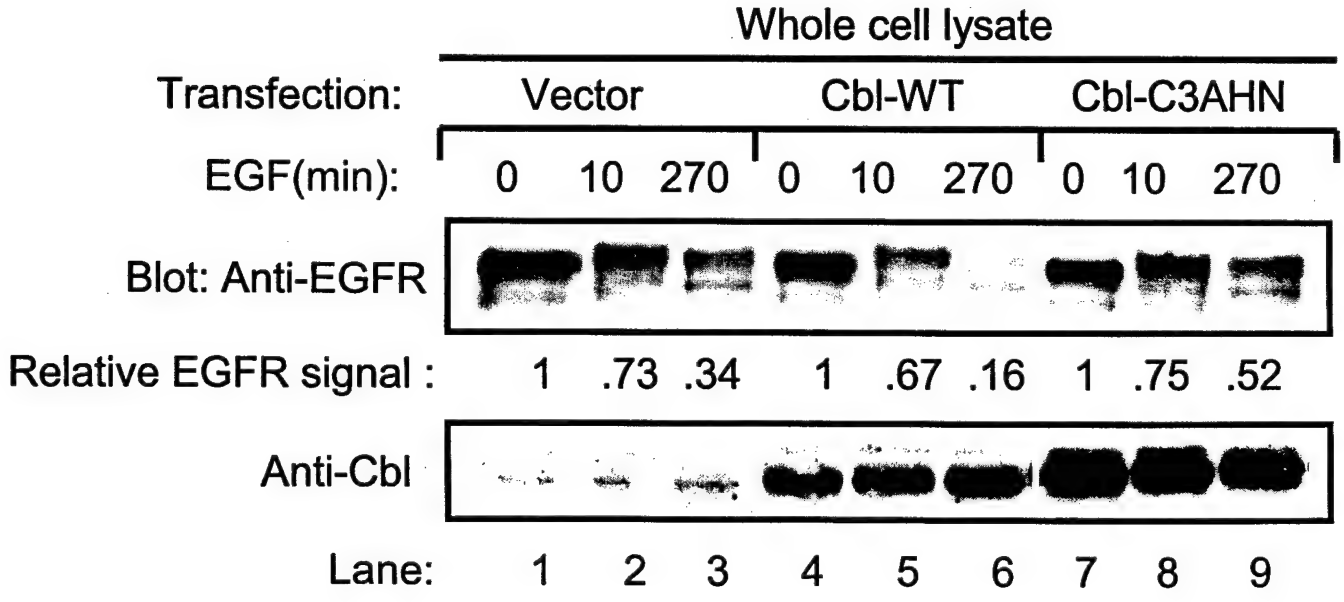

Whole cell lysate
Transfection:
Vector
Cbl-WT
Cbl-C3AHN
EGF(min):
0 10 270 0 10 270 0 10 270
Blot: Anti-EGFR
Relative EGFR signal :
1 .73 .34 1 .67 .16 1 .75 .52
Anti-Cbl
Lane:
1 2 3 4 5 6 7 8 9

Fig. 5 A

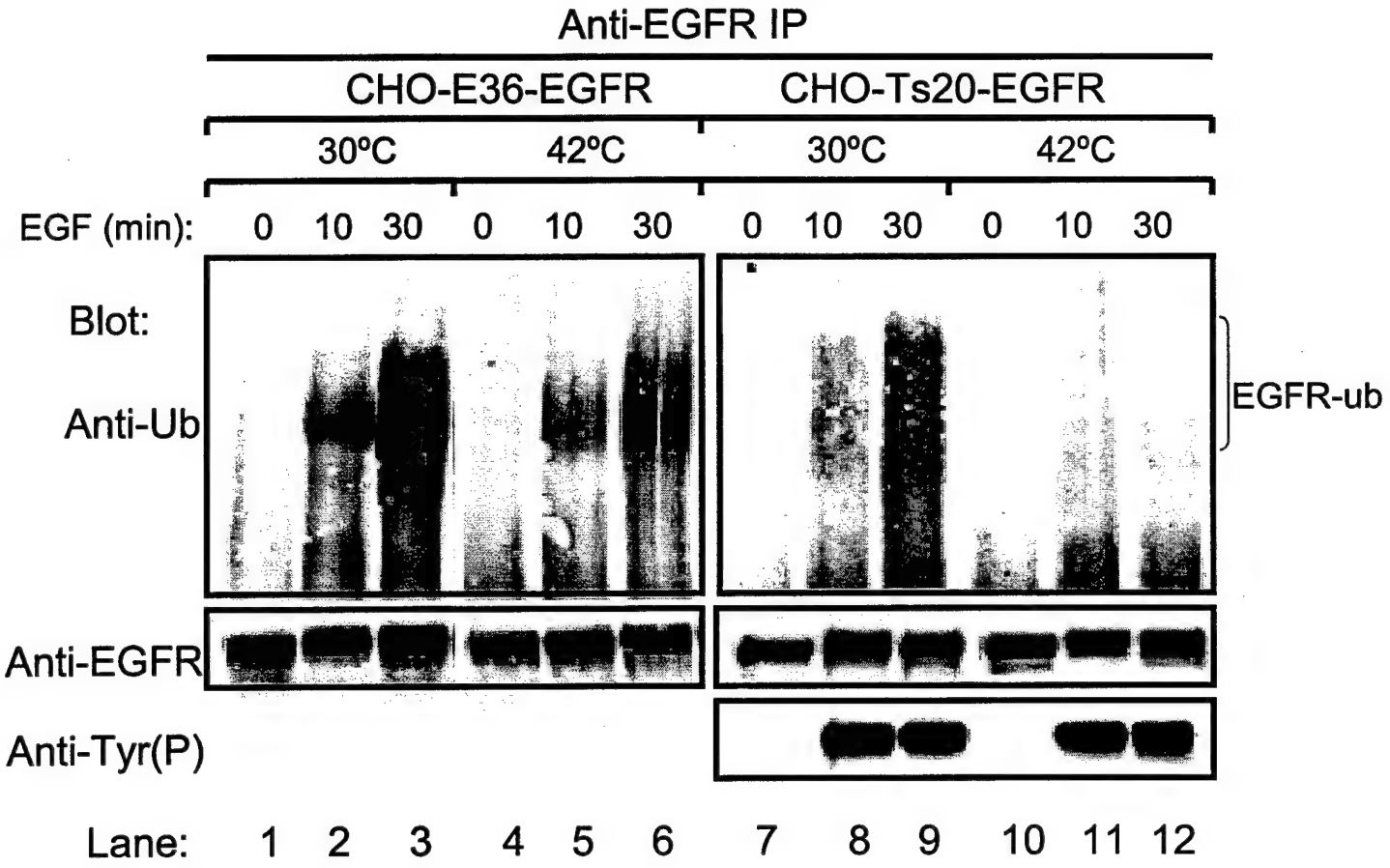

Anti-EGFR IP
CHO-E36-EGFR
CHO-Ts20-EGFR
30°C
42°C
30°C
42°C
EGF (min):
0 10 30 0 10 30 0 10 30 0 10 30
Blot:
Anti-Ub
EGFR-ub
Anti-EGFR
Anti-Tyr(P)
Lane:
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 5 B

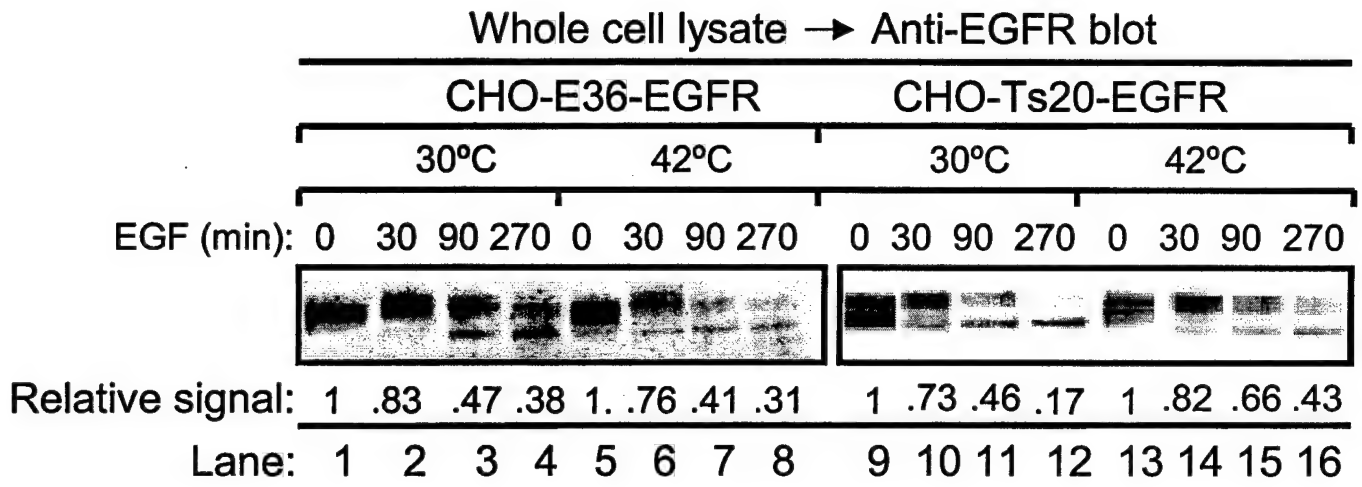

Whole cell lysate → Anti-EGFR blot
CHO-E36-EGFR
CHO-Ts20-EGFR
30°C
42°C
30°C
42°C
EGF (min): 0 30 90 270 0 30 90 270 0 30 90 270 0 30 90 270
Relative signal: 1 .83 .47 .38 1. .76 .41 .31 1 .73 .46 .17 1 .82 .66 .43
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 6

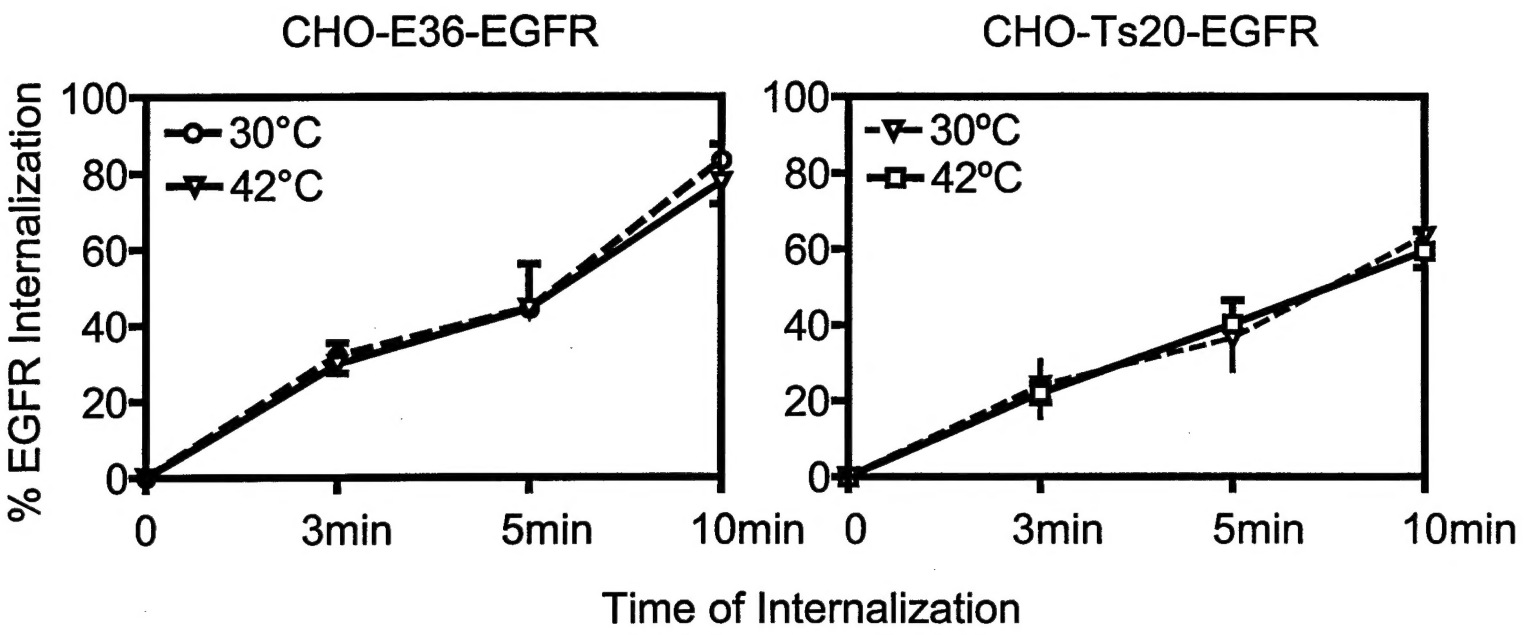

CHO-E36-EGFR
CHO-Ts20-EGFR
% EGFR Internalization
100
80
60
40
20
0
30°C
42°C
30ºC
42ºC
0
3min
5min
10min
Time of Internalization

Fig. 7A

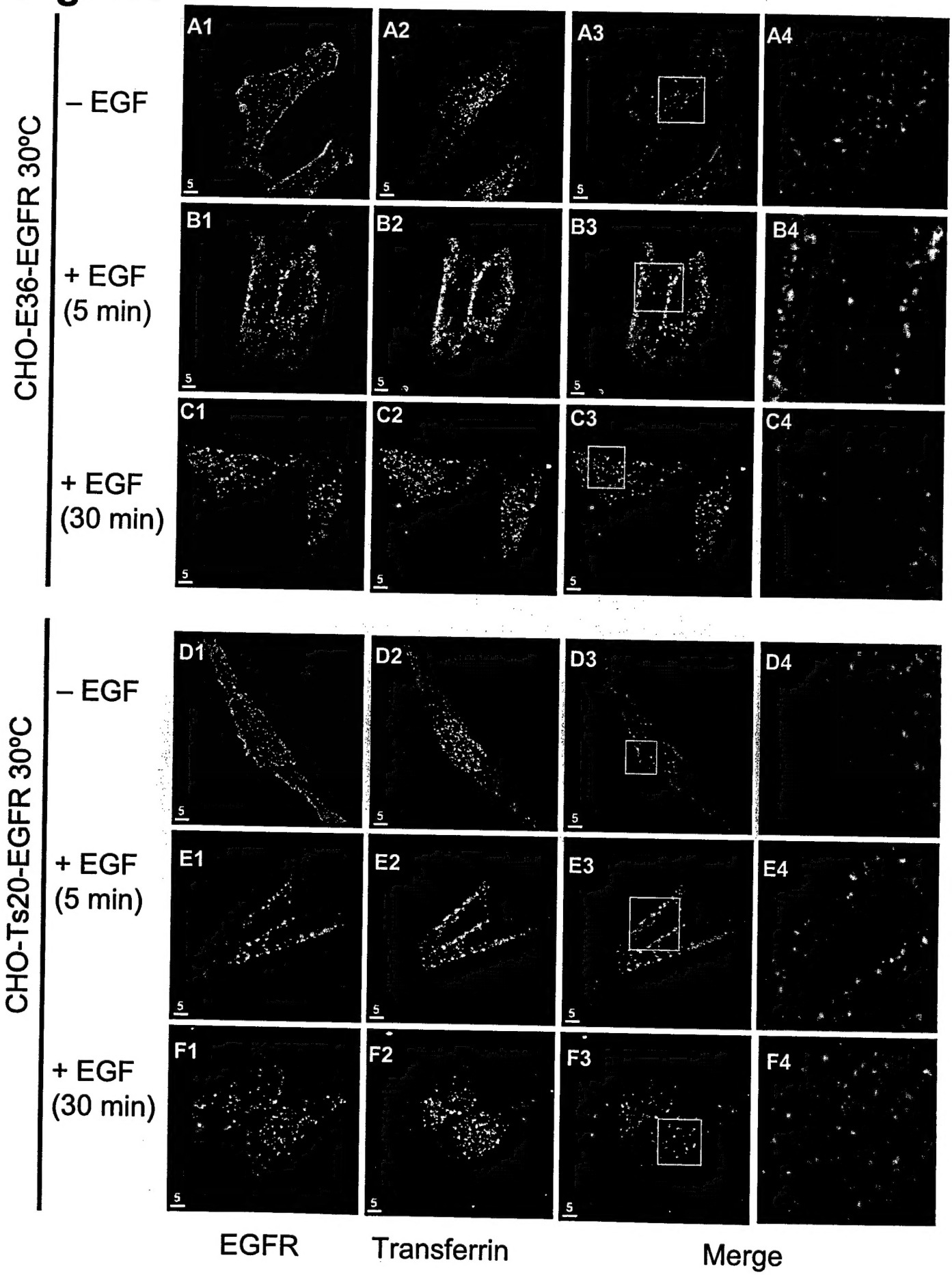

CHO-E36-EGFR 30°C
– EGF
+ EGF (5 min)
+ EGF (30 min)
CHO-Ts20-EGFR 30°C
– EGF
+ EGF (5 min)
+ EGF (30 min)
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4
F1
F2
F3
F4
5
EGFR
Transferrin
Merge

Fig. 7B

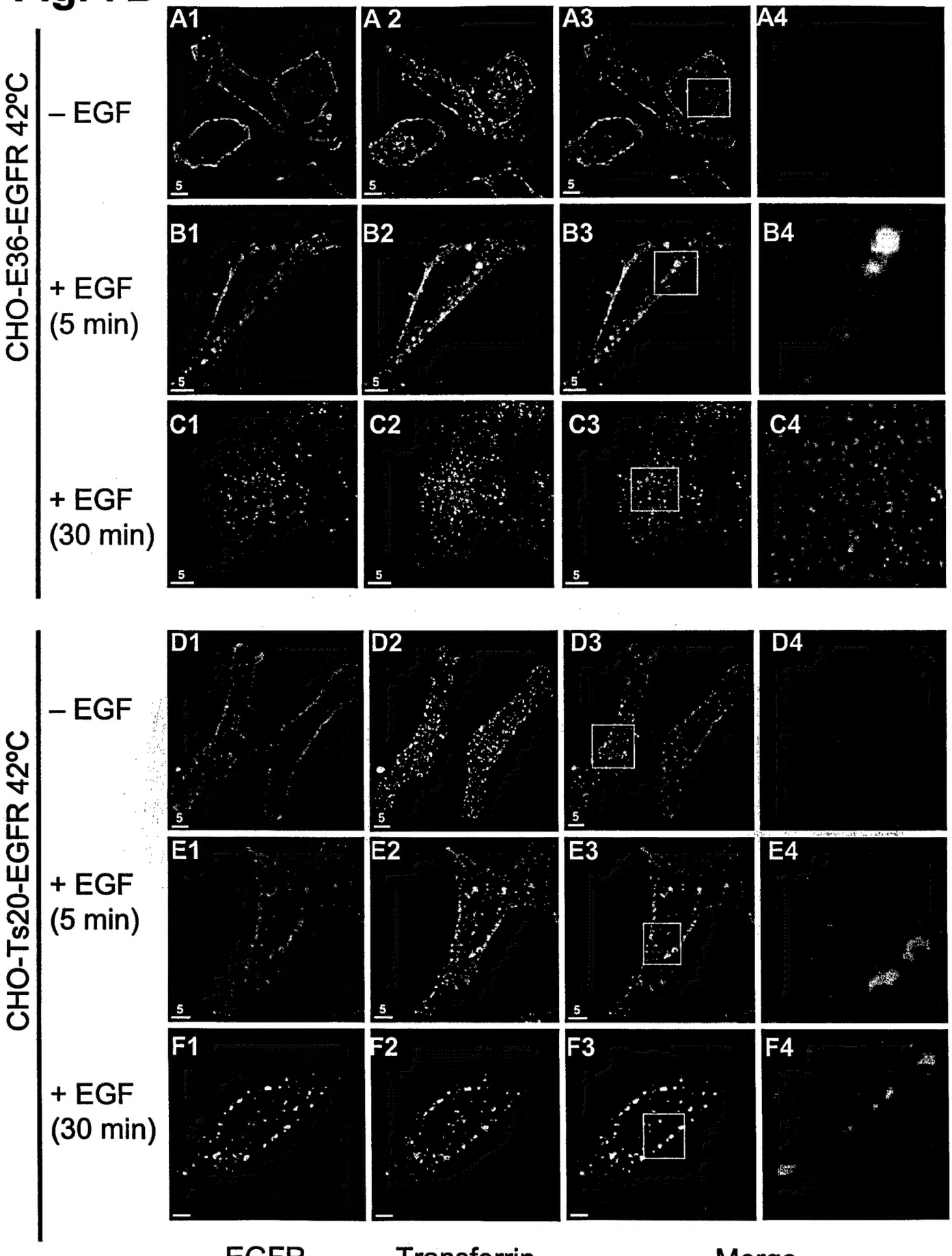
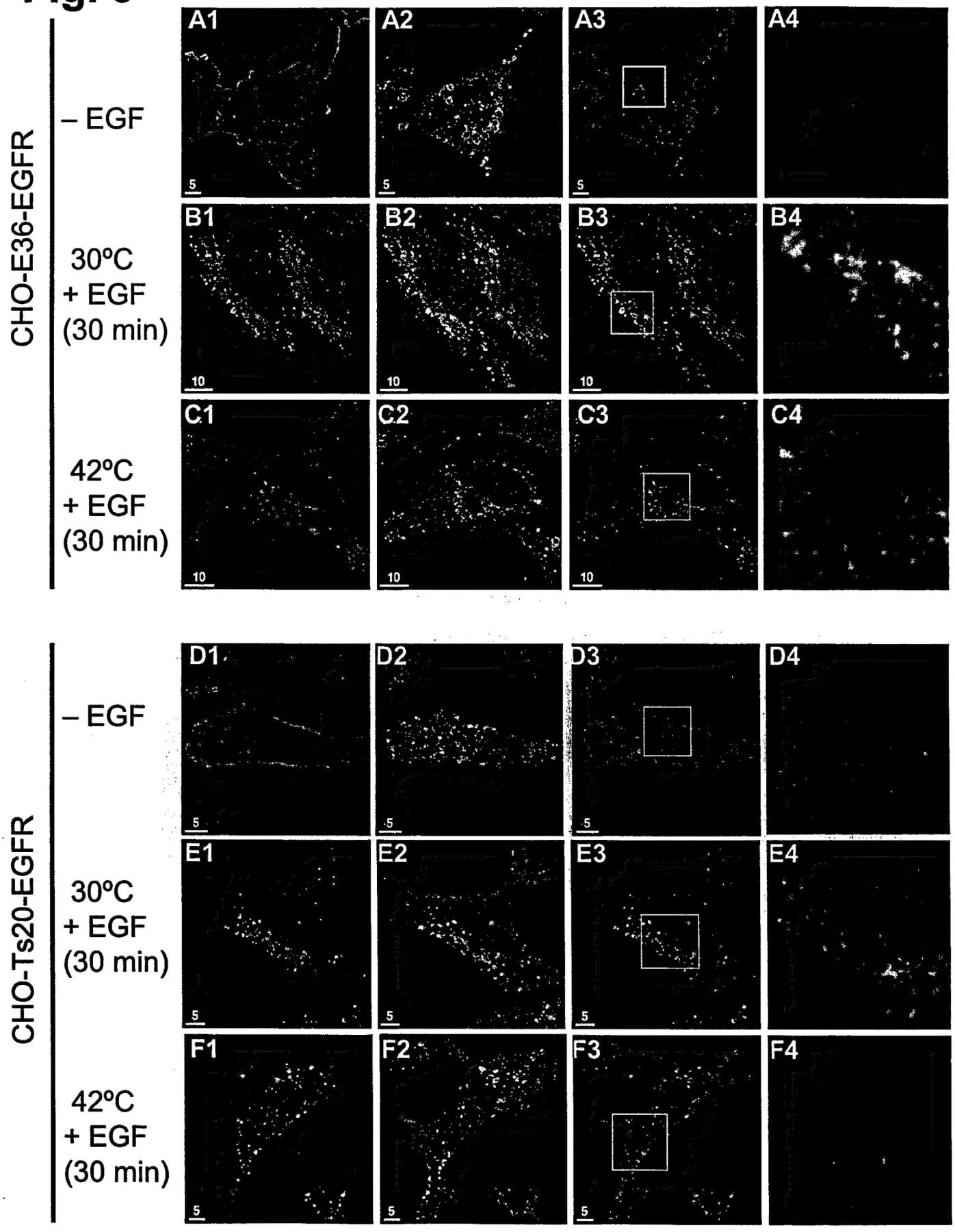

CHO-E36-EGFR 42°C
– EGF
+ EGF (5 min)
+ EGF (30 min)
A1
A 2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
CHO-Ts20-EGFR 42°C
– EGF
+ EGF (5 min)
+ EGF (30 min)
D1
D2
D3
D4
E1
E2
E3
E4
F1
F2
F3
F4
EGFR
Transferrin
Merge

Fig. 8


CHO-E36-EGFR
– EGF
30°C
+ EGF
(30 min)
42°C
+ EGF
(30 min)
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
CHO-Ts20-EGFR
– EGF
30°C
+ EGF
(30 min)
42°C
+ EGF
(30 min)
D1
D2
D3
D4
E1
E2
E3
E4
F1
F2
F3
F4
EGFR
LAMP-1
Merge